UNIVERSIDAD NACIONAL DE COLOMBIA

Extracellular polymeric substances (EPS) production in *Sulfobacillus thermosulfidooxidans* and its relevance on attachment to metal sulfides.

Mauricio Aguirre Morales

Universidad Nacional de Colombia
Facultad de Ciencias
Maestría interfacultades en Microbiología
Bogotá, Noviembre de 2012

Extracellular polymeric substances (EPS) production in *Sulfobacillus thermosulfidooxidans* and its relevance on attachment to metal sulfides.

Mauricio Aguirre Morales

Tesis presentada como requisito parcial para optar por el titulo de:

Magister en ciencias-Microbiología

Director:

Prof. Dr. Wolfgang Sand

Aquatische Biotechnologie

Biofilm centre

Universität Duisburg-Essen

Codirectora:

Dr. Catalina Arévalo Ferro
Profesora
Departamento de Biología
Universidad Nacional de Colombia

Línea de investigación:

Microbiología ambiental

Universidad Nacional de Colombia
Facultad de ciencias
Bogotá noviembre de 2012

“If you focus your mind on the freedom and community that you can build by staying firm, you will find the strength to do it.”

Richard Stallman

Acknowledgements

This work was carried out in the Biofilm center of the Universität Duisburg-Essen, many thanks to all the people in this institution who helped me to develop this project.

Special thanks to Mario Vera and Felipe Leon whose academic and personal support became essential during the project. Thanks to my family and friends who believed in me and gave me the strength to carry on during difficult times.

TABLE OF CONTENTS

List of figures

List of tables

List of abreviations

EPS	Extracellular polymeric substances
CLSM	Confocal laser scanning microscopy
DSM-DSMZ	Deutsche sammlung von mikroorganismen und zellkulturen (German collection of microorganisms and cell culture).
ABC	ATP binding Cassette.
AMD	Acid mine drainage.
CBB	Calvin-Benson-Bassham.
RuBisCo	Ribulose-1,5-bisphosphate carboxilase oxygenase.
PEP	Phosphoenolpyruvate carboxylase.
ATP	Adenosine triphosphate.
GTF	Glycosyltransferases.
Mac	Mackintosh basalt salt medium.
DAPI	4´.6-diamidino-2-phenylidole.
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid.
TRITC	Rodhamine.
FITC	Fluorescein isothiocynate.
ConA	Concanavalin A.
PNA	Peanut agglutinin.
ECA	Erythrina cristagally.
SBA	Soybean agglutinin.
UEAI	Ulex europeaus agglutinin I.
PWM	Pokeweed miotgen.
BSI	Bandeiraea simplicifolia isolectin.
PHAE	Phaseolus vulgaris agglutinin E.
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol.

BSA	Bovine serum albumin.
PBS	Phosphate buffered saline.
G6PDH	Glucose 6-phosphate dehydrogenase.
SDS	Sodium dodecyl sulfate.
EDTA	Ethylenediaminetetraacetic acid.
DNAse	Deoxyribonuclease.
RNAse	Ribonuclease.
RPM	rounds per minute.
COG	Closter of orthologous groups.
KEGG	Kyoto encyclopedia of genes and genomes.
KO	KEGG orthologie.
PCG-CTP	Protein coding genes coding transmembrane proteins.
KMT	Protein coding genes connected to KEGG pathways-membrane transport pathway.
PCGFP	Protein coding genes with function prediction.
BLAST	Basic local alignment search tool.
NCBI	National center for biotechnology information.
PCR	Polymerase chain reaction.
RT-PCR	Real time PCR.
EMBOSS	European molecular biology open software suite.
Coll	Colloidal fraction.
WF	Washed fraction.
CFE	Capsular first extraction fraction.
CSE	Capsular second extraction fraction.
NC	Negative control.

1. Resumen

La extracción de metales a partir de minerales azufrados ha sido un paso importante para la industria minera a través de los años. Hay microorganismos capaces de crecer en zonas mineras y depósitos de menas, utilizando compuestos presentes en la menas para obtener energía, precipitando de esta forma otros compuestos presentes en las mismas. El Uso de microorganismos biolixiviadores acidofilos en un proceso llamado biohydrometalurgia, se ha convertido en una alternativa a la minería convencional que permite la extracción de metales a partir de menas. La biohydrometalurgia se ha mostrado como una alternativa amigable al medio ambiente y más económica en comparación con la minería convencional. En la naturaleza los microorganismos son capaces de construir estructuras como los *biofilms*, los cuales les confieren a los microorganismos resistencia a diferentes condiciones ambientales adversas. Estos son comunidades de microorganismos embebidos en sustancias poliméricas extracelulares (EPS). El EPS permite un espacio de reacción bioquímica; en microorganismos lixiviadores se ha observado que el *biofilm* compuesto por EPS juega un papel fundamental en la degradación de menas. *Sulfobacillus thermosulfidooxidans* es un microorganismo termófilo moderado utilizado en biolixiviacion, sin embargo poco se sabe sobre *S. thermosulfidooxidans* y la naturaleza y composición del EPS producido por el mismo. La implementación de *S. thermosulfidooxidans* en procesos de biominería, es prometedora debido a que la biominería es un proceso exotérmico donde se alcanzan altas temperaturas. Con el objetivo de utilizar *S. thermosulfidooxidans* en procesos de biominería se estudio su crecimiento en presencia de diferentes fuentes de energía, la composición de EPS y mediante métodos bioinformaticos, algunos genes potencialmente involucrados en el proceso de producción de EPS. El crecimiento de *S. thermosulfidooxidans* cambia de acuerdo a la fuente de energía que se implemente en el medio, la máxima concentración celular que se alcanzo fue con el medio con sulfato de hierro como única fuente de energía. *S. thermosulfidooxidans* es capaz de crecer bajo condiciones mixotroficas y heterotróficas, sin embargo su crecimiento bajo condiciones mixotroficas es más alto

en comparación con condiciones heterotróficas. Adhesión directa a pirita y azufre se siguió a través del tiempo mediante microscopia confocal (CLSM). La adhesión a pirita parece ocurrir de manera más rápida puesto que sobre esta superficie se puede observar aglomeración celular sobre espacios reducidos, en tiempos más tempranos en comparación con la adhesión al azufre donde se observo en tiempos posteriores. Se extrajo y analizo el EPS producido por células de *S. thermosulfidooxidans* crecidas en presencia de azufre, pirita y sulfato de hierro. Se determinaron algunos de los componentes del EPS, la proporción de los componentes cambia de acuerdo a la fuente de energía que se utilice para crecer el microorganismo y los ácidos húmicos se encontraron en mayor proporción. Se observo también que la producción de EPS se ve disminuida en el caso de las células en estado planctónico en términos de peso seco del EPS y en algunos casos fue inclusive indetectable. Con el objetivo de visualizar e identificar algunos de los componentes del EPS, se realizaron tinciones con lectinas; se observo que la composición del EPS no solo cambia dependiendo de la fuente de energía que se utilizo, azufre o pirita, para crecer el microorganismo sino también del estado celular en el que se encuentre, sésil o planctónico. Solamente con concanavalina A se obtuvo una interacción positiva bajo todas las condiciones probadas. Genes previamente reportados involucrados en la síntesis de EPS y formación de *biofilm*, fueron buscados en el genoma de *S. thermosulfidooxidans* DSM 9293, sin embargo no se obtuvieron asignaciones verdaderas. Por lo tanto, secuencias de proteínas relacionadas con los mecanismos de producción y exportación de EPS fueron buscados en el genoma y se encontraron secuencias relacionadas con transportadores ABC las cuales se seleccionaron para su posible amplificación. Sin embargo no se obtuvo amplificación satisfactoria que permitiera medir los niveles de expresión de estos genes mediante PCR en tiempo real.

Palabras clave: biolixiviacion, sustancias poliméricas extracelulares, *biofilm*, *Sulfobacillus thermosulfidooxidans*.

2. Summary

Retrieving metals from sulphide minerals has been a key step for the mining industry through many years. There are microorganisms that colonize mining areas and ore deposits and are able to use some of the compounds of the ores to obtain energy, precipitating some other components. The use of acidophilic leaching bacteria, in a process known as biohydrometallurgy, has become an alternative way to traditional mining allowing the recovery of metals. Biohydrometallurgy has shown to be environmentally friendly and it has shown to be cheaper compared to conventional mining (pyrometallurgy). In nature, microorganisms are able to build structures such as biofilms that confer resistance to microorganisms under adverse environmental conditions. Those are communities of microorganisms, embedded in extracellular polymeric substances (EPS). The EPS also enhances a reaction space by extracellular biochemical reaction; in leaching organisms it has been observed that biofilms composed of EPS play a key role in degradation of ores. *Sulfobacillus thermosulfidooxidans* is a moderate thermophile used in bioleaching, nevertheless little is known about *S. thermosulfidooxidans* and the nature and composition of its EPS. The use of *S. thermosulfidooxidans* in biomining is promising because it is an exothermal reaction and it reaches high temperatures. With the idea of using this bacterium in the future for biomining process, its growth was studied in the presence of different energy substrates, the composition of its EPS and by bioinformatics, some genes potentially involved in the process of EPS production. The growth of *S. thermosulfidooxidans* changes according to the energy source used in the medium, the maximum cell concentration was achieved in medium with iron sulphate as energy source. *S. thermosulfidooxidans* is able to grow under heterotrophic and mixotrofic conditions, nevertheless growth under mixotrophic conditions is higher than under heterotrophic conditions. Attachment to sulfur and pyrite surfaces was followed by Confocal Laser Scaning Microscope (CLSM) along time. Attachment to piryte surfaces seems to be faster since cell agglomeration over reduced spaces can be seen earlier than in sulfur surfaces. The EPS produced by *S. thermosulfidooxidans*

grown in different energy sources, sulfur, pyrite and iron sulphate was extracted and analyzed. The EPS composition was determined, the proportion of its components changed according to the energy source and humic acids were found to be one of the major components. It was observed that EPS production diminishes in the planktonic culture and it was not detectable in some cases. Staining with labeled lectins was made in order to visualize and identify some components of its EPS; it was observed that also the components of the EPS changes not only depending on the energy source tested, pyrite and sulfur, but also in the planktonic and sessile state of the cells. Only concanavalin A had a positive interaction under all the conditions tested. Genes previously reported to be involved in EPS synthesis and biofilm formation, were searched on *S. thermosulfidooxidans* DSM 9293 genome but no meaningful matches were found. Thus, sequences coding for proteins related to the mechanisms of production and exportation of EPS were searched on the genome and ABC transporters were found and selected for amplification. However, no successful amplification or measurement of level of expression of these genes was achieved.

Keywords: bioleaching, extracellular polymeric substances, biofilm, *Sulfobacillus thermosulfidooxidans*.

3. Theoretical framework

3.1. Biomining and bioleaching

Biomining is known as the industrial process of extracting valuable minerals from ores with the help of microorganisms. There are several ways microorganisms can promote extraction of target minerals from ores; these depend on the microorganism and the ore. An indirect mechanism in the leaching process has been proposed which involves the production of ferric iron or protons which chemically attack the ore and cause its dissolution. Namely cooperative leaching occurs when microorganisms produce these protons. On the other hand, a direct mechanism involves physical contact between the ore and the cell membrane causing leaching by enzymatic activity. An important aspect of bioleaching mediated by direct contact between microorganisms and the ore is the production of EPS by the microorganism which helps to attach to the ore surface and it also helps to leach it (Gehrke et al., 1998b; Rawlings, 2002). In Fig. 1, the different mechanisms by which a microorganism can achieve leaching of pyrite are shown.

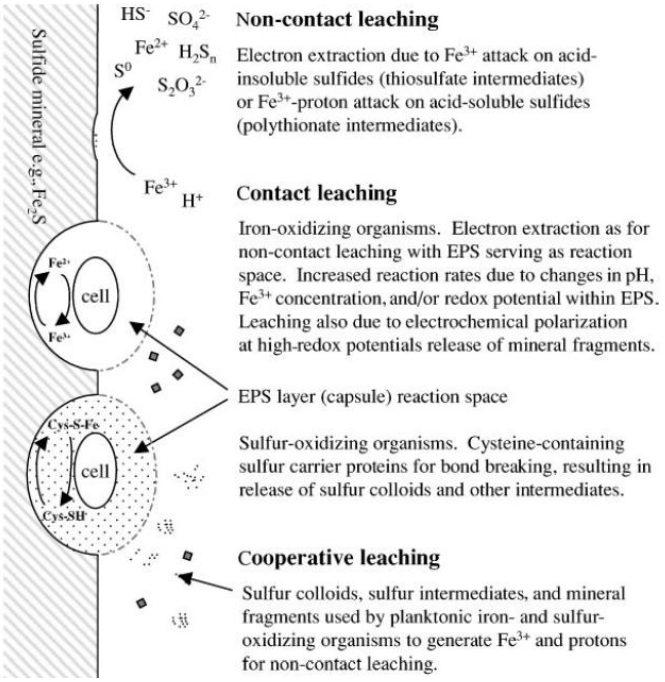


Figure 1: Oxidation mechanisms of pyrite followed by microorganisms(Rawlings, 2002).

Biomining has become an economically attractive way to retrieve minerals since 1988 (Blake II et al., 1994), and there are different processes where biomining is performed. These processes involve the use of several types of bioreactors where bioleaching can be performed such as irrigation-type and stirred tank-type, the conditions and microorganisms are different and these differences are due to the ore that is being leached (Rawlings, 2002).

3.2 Bioleaching microorganisms and Acid Mine Drainages (AMD)

There are microorganisms inhabiting ores and mining areas which are exposed to atmospheric conditions. Most of them are obligate acidophilic chemolithotrophs which use oxygen to oxidize sulfur and/or ferrous iron from ores using them as electron donors in order to obtain energy. These properties have allowed them to be used in biomining processes (Ghauri et al., 2007).

Leaching environments have been observed to contain low microbial diversity (Kock & Schippers, 2008; Nicomrat et al., 2006; Schippers & Sand, 1999; Mendez et al., 2008). This is due to the few types of substrates available and extreme conditions for microbial growth. The main acidophilic leaching bacteria described belong to the genera *Acidithiobacillus, Leptospirillum, Sulfobacillus* and *Acidiphilium* while among the leaching archaea the genera *Ferroplasma*, *Methalospera*, *Acidianus* and *Sulfolobus,* are found, being the last three ones thermophiles. One of these leaching environments is known as Acid Mine Dreinage (AMD), which is characterized by their very acid conditions and high concentration of toxic heavy metals (Brockmann et al., 2010). In Fig. 2 the microbial diversity of soil samples from mining area and a soil control is shown in terms of percentage.

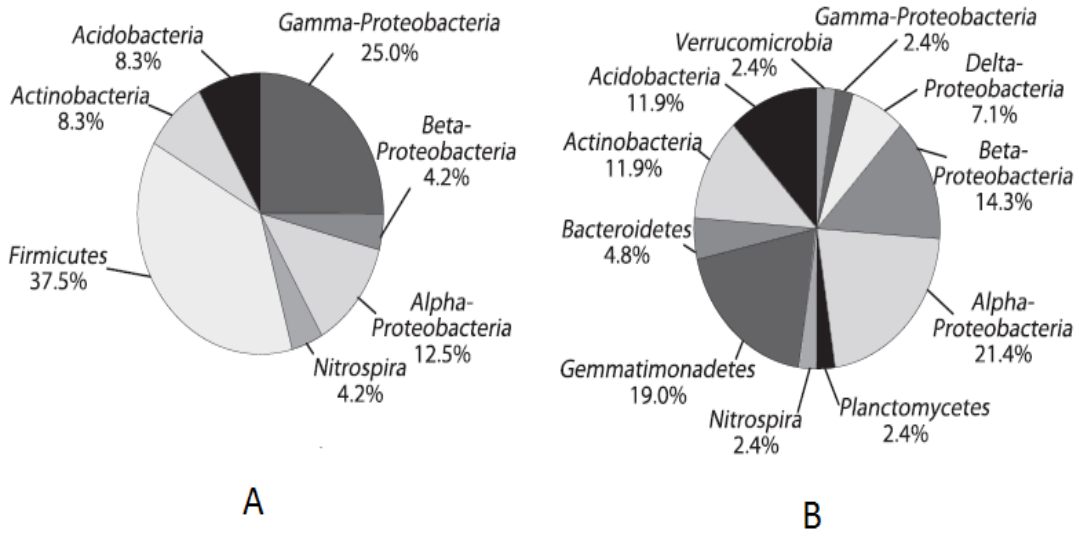


Figure 2: Distribution of phylotypes from two different soil samples, A is a soil sample from a mine and B is a soil sample taken as a control from a non mining soil, (Mendez et al., 2008).

Acidithiobacillus ferrooxidans is commonly found in acid environments such as AMD and mining dumps and it is a clear example of the microorganism that can be found in these environments (Schrenk et al., 1998). It is a mesophilic acidophilic microorganism widely used in bioleaching process of metal ores. It is a chemolitoautothropic Gram (-) bacteria which obtains energy from the oxidation of different compounds such as ferrous iron, elemental sulfur and other partially oxidized sulfur compounds (Chi et al., 2007). Nevertheless it is mesophile, requiring longer times for bioleaching with oxidation rates below some thermophilic organisms (Ding et al., 2007). *Acidithibacillus thiooxidans* belongs to the same genera, and is also widely used in this kind of processes (Bevilaqua et al., 2002)

The same bacteria used for biomining can be also used for bioleaching processes where AMD is treated, which usually includes heavy metal remotion and pH rising (Bond et al., 2000).

3.3 *Sulfobacillus* spp.

Sulfobacillus are chemolithotrophs, Gram (+), aerobic, non-motile facultative autotrophic bacteria able to use ferrous ion and elemental sulfur as energy source

(Karavaiko et al., 1990). Nevertheless the growth on iron is better than in sulfur (Egorova et al., 2004). It contains the necessary metabolic enzymes to grow under heterotrophic, mixotrophic and autotrophic conditions (Krasil'nikova et al., 2010; Egorova et al., 2004). Although for *in vitro* autotrophic growth it needs to be supplemented with CO_2 and heterotrophic growth is not as high as under the other conditions (Krasil'nikova et al., 2010; Caldwell et al., 2007)

Sulfobacillus thermosulfidooxidans is an acidophilic, endospore-forming bacterium which is able to synthesize flagella (Muravyov et al., 2010). It is widely found in bioleaching process, being a moderate thermophile (40°-60°), it is considered to be more effective for leaching purposes (Ding et al., 2007). Cells occur in chains of up to four cells, rods can be straight or slightly curved (Bogdanova et al., 2006).

Autotrophic growth of *S. thermosulfidooxidans* with ferrous iron has only been seen in the presence of CO_2, fixing it through Calvin-Benson-Bassham (CBB) cycle with the help of the RuBisCo enzyme and PEP carboxilase (Caldwell et al., 2007; Clark & Norris, 1996; Krasil'nikova et al., 2010). Under these conditions *S. thermosulfidooxidans* requires very high amounts of CO_2 and also the presence of yeast extract; in the absence of oxygen it can also grow using ferric iron as electron acceptor and organic or inorganic sulfur compounds as electron donors (Bridge & Johnson, 2000). The growth of *S. thermosulfidooxidans* is limited by the formation of ferric ion (Becker et al., 2011).

S. thermosulfidooxidans is able to oxidize up to 90% of the available iron in the medium during the first 30 h, furthermore when it is attached to a surface this percentage is higher and it is achieved in shorter times (Ding et al., 2007). Nevertheless bioleaching mechanisms and EPS production in *S. thermosulfidooxidans* has not been studied carefully and there are not many reports about in this topic concerning this bacteria.

3.4 Extracellular polymeric substances and biofilm formation

Biofilms are communities of microorganisms which are attached to a surface (O´Toole et al., 2000). There are different stages in the process of biofilm formation and these different stages are achieved by different methods depending on the microorganism. Initially there is a first physical contact between the bacteria and the surfaces that could be mediated by flagella or pilli. After this initial contact the cells start to produce adhesins to attach to the surface and later on the synthesis of these adhesins becomes specific to the surface where the cells are attaching to (Karatan & Watnick, 2009). Among these adhesins, extracellular polymeric substances (EPS) can be found. EPS conforms a gelatinous matrix which represents between 60 and 90% of the dry weight of the biofilm while the microorganisms dry weight is around 10 % (Flemming & Wingender, 2010). These structures allow microorganisms to subsist under extreme conditions like AMD (Brockmann et al., 2010).

It has been observed that the EPS layer on a surface increases its thickness along with pH dropping in the case of *S. thermosulfidooxidans*. This suggest that the layer may prevent cell lysis by giving coverage and allowing the cells to survive pH even under 0.9 (Becker *et al*., 2011). Also there is evidence pointing that attached microorganisms have different leaching rates versus microorganisms on planktonic states (Blake II et al., 1994).

The EPS is mainly composed by two fractions clearly defined as tightly and loosely bound fractions. The tightly bound is the EPS which is strongly and directly stuck to the cell membrane while the loosely bound EPS is the previously tightly bound EPS which later is detached by the new synthesized EPS and therefore is no longer in direct contact with the cell membrane (Li & Yang, 2007). The loosely bound EPS is easily removed from a cell culture by centrifugation while the tightly bound requires special procedures.

The EPS may change its composition depending on the surface where the microorganism is trying to attach and also on the strain that is producing it (Dazzo & Brill, 1979; Chae & Schraft, 2000).

3.4.1 Role of EPS in attachment

The cell attachment, mediated by EPS, and further biofilm formation on metal sulfides by acidophilic microorganisms plays a fundamental role in bioleaching and oxidation processes (Gehrke et al., 1998b; Sampson et al., 2000). The adhesion of microorganism to substrates and supports has been explained as hydrophobic and electrical interactions between bacteria and surface (Arredondo et al., 1994). Lewis acids forces and van der Waal´s forces are some of the interactions between EPS and surface (Gehrke et al., 1998b). The properties by which the cell attaches to the surface are conferred by EPS. Some microorganisms EPS mutants have shown to present lower attachment and further biofilm maturation is also different compared to wild strains (Watnick & Kolter, 1999).

For bioleaching microorganisms which EPS has been extracted or removed the adhesion time to ore surfaces is longer and adhesion rates are lower than cells which are still with their EPS (Arredondo et al., 1994; Sand et al., 1995). Nevertheless it has been observed that for some microorganism the synthesis of new EPS, if removed, is made in a short time after incubation in the presence or ores (Gehrke et al., 1998b).

For adhesion to roots by symbiotic microorganisms, it has been observed that EPS extracted from cells can bind itself to the roots, therefore playing a key role (Dazzo & Brill, 1979). Polysaccharides present in the EPS produced by these microorganisms bind in some cases to lectins present in some parts of the plant, allowing microorganisms to grow and build biofilms (Dazzo & Brill, 1979). In the case of bioleaching microorganisms it has been observed also that EPS extracted from cells binds to the ore surface and furthermore it can outcompete cells for adhesion space (Arredondo et al., 1994).

In the case of *S. thermosulfidooxidans* it has been observed that it produces EPS in order to attach to pyrite surfaces and the EPS remains stuck on the surface even in some cases when the cell gets detached (Becker et al., 2011).

3.4.2 Mechanisms of EPS synthesis

EPS production occurs under stressful conditions in some cases and the nature of the surface where the cells are trying to grow is also an important variable. Nevertheless the part of the EPS known as tightly bound, is produced all over the cell cycle no matter if cells are in the presence of a support or not (Li & Yang, 2007).

Synthesis of EPS may be done by different pathways; there are currently three of these pathways described. The first involves the Wzy/Wzx complex where the protein Wzx produces monomers, the Wzy polymerizes them and finally the Wza is a transmembrane channel. In this pathway, the polymerization is regulated by a polysaccharide copolymerase. The second one is related to the ATP binding cassettes (ABC). ABC transporters are important for cell aggregation which is an initial step on biofilm formation (O´Toole et al., 2000). This complex is composed of a transmembrane domain and a nucleotide membrane which binds to ATP changing the conformation of the structure allowing molecules to get through; the polymerization process is regulated by glycosiltransferses. The third one is the synthase depended pathway which mechanisms are unknown. All the mechanisms are related to antigens synthesis and belong to the different secretion systems known in bacteria, making them very similar among all (Cuthbertson et al., 2010; Whitfield, 2006). In Fig 3 a scheme of the different mechanisms is shown.

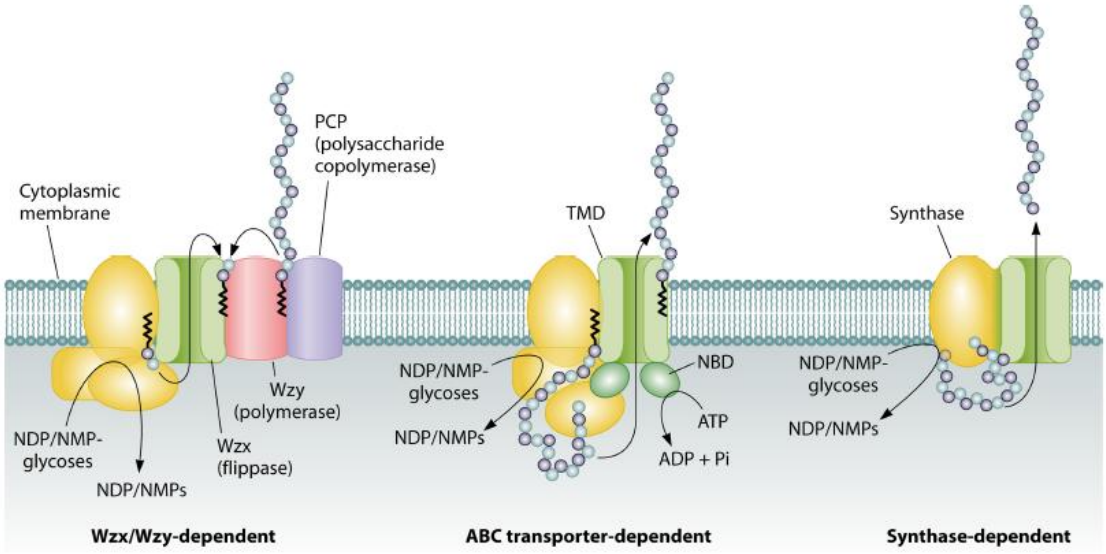


Fig 3. different pathways of EPS synthesis (Cuthbertson et al., 2010).

The production of polysaccharides for EPS on Gram (-) bacteria may be described within 5 steps, the first step is the formation of glucose 1-P or glucose 6-P, these compounds can be achieved via three different pathways. After the glucose 1-P and 6-P is available it suffers a modification to obtain UDP-Galactose, rTDP-Rhammnose, UDP-glucose and GDP-mannose. The third step involves the production of a lipid anchor to the inner membrane where, in step four, the sugars are attached on the polymerization process. Finally the molecule is inverted and exported to the outskirts of the cell where it attaches to the outer membrane. The polymerization is carried out by glycosyltransferases (GTFs) which also determine the length of the polymer (Barreto *et al*., 2005). The EPS composition in acidophilic bioleaching microorganisms changes in response to the substratum/substrate on which the organisms are growing (Sand & Gehrke, 2006). Thus, it is necessary to know which nutrients, sugars and other compounds present in the media have an effect on the formation and conformation of EPS. However, general information on the polymerization pathway is little and also the whole process in Gram (+) bacteria. A model of polymerization pathway for Gram (-) bacteria is shown in Fig 2.

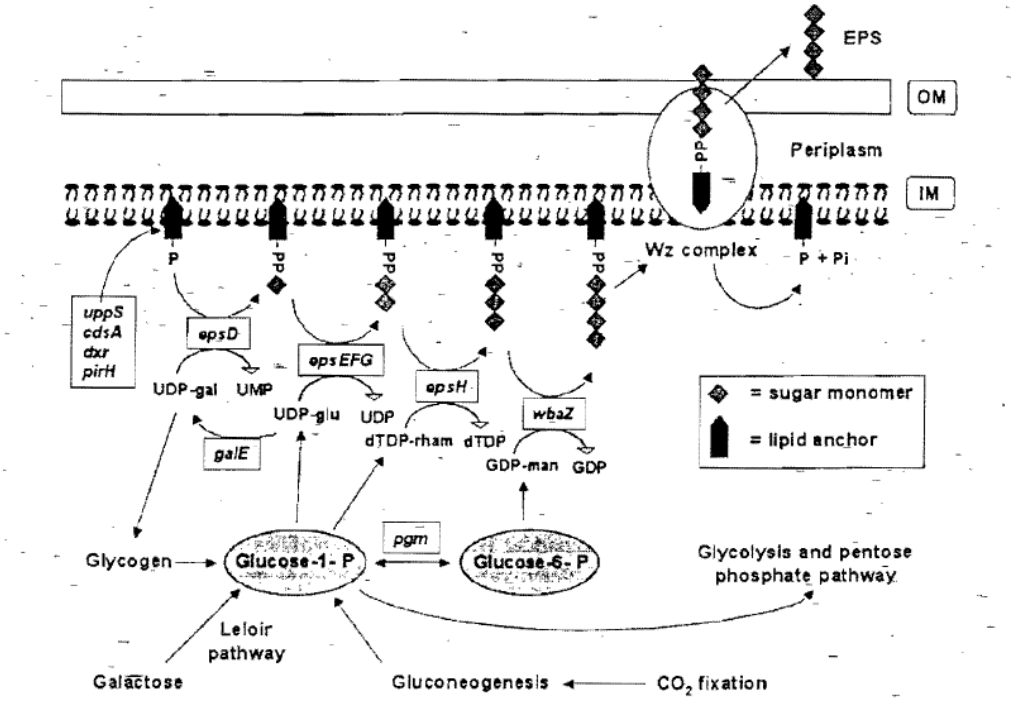


Figure 4 polymerization pathway of EPS on Gram (-). (Taken from: Unexpected insights into biofilm formation by *Acidithiobacillus ferrooxidans* revealed by genome analysis and experimental approaches. 16th International Biohydrometallurgy Symposium , 817-825. 2005.

4. Objectives

4.1. Main objective.

The main goal of this work was to establish the influence and variations in extracellular polymeric substances (EPS) production in *Sulfobacillus thermosulfidooxidans* in response to the presence of different substrata by microscopy, EPS analysis and measurement of expression level of some genes potentially related to their biosynthesis.

4.2. Specific objectives.

- To establish the influence of different energy substrates on the growth and EPS production by *S. thermosulfidooxidans*.

- To determine the composition of *S. thermosulfiddoxidans* EPS.

- To determine which lectins can recognize polysaccharides from *S. thermosulfidooxidans* EPS related to their process of biofilm formation.

- To identify through bioinformatics methods potential genes involved in EPS biosynthesis in *Sulfobacillus*, using the available genome secuence of *S. thermosulfidooxidans*, for eventually measuring their expression levels under different biofilm formation conditions.

5. Methods

5.1. Culture media and growth conditions.

The strain used in this thesis was *Sulfobacillus thermosulfidooxidans* DSM 9293 initially isolated by Karavaiko and collaborators in Moscow in 1978 from a mining area in Kazakhstan (Karavaiko et al., 1990; Tourova et al., 1994; 1991). *S. thermosulfidooxidans* is able to grow in heterotrophic, mixotrophic and autotrophic conditions (Egorova et al., 2004; Krasil'nikova et al., 2010). It is able to grow using iron and sulfur as energy sources, thus is able to grow with metal sulfides (Caldwell et al., 2007); nevertheless the growth on iron is higher than in sulfur (Egorova et al., 2004).

S. thermosulfidooxidans was grown on Mackintosh basal salt solution (Mac): $(NH_4)SO_4$ 0.2 mM; KH_2PO_4 1 mM; $MgCl_2$ 125 µM; $CaCl_2$ 1 mM; $MnCl_2$ 0.5 µM; $ZnCl_2$ 0.5 µM; $CoCl_2$ 0.5 µM; H_3BO_3 0.5 µM; $NaMoO_4$ 0.05 µM; $CuCl_2$ 0.5 µM; H_2SO_4 9.6 mM and $FeSO_4$ 180 mM, pH was adjusted to 3 (Mackintosh, 1978), the protocol was modified from the original and the KH_2PO_4 was added at a higher concentration (5 mM).

Heterotrophic and mixotrophic conditions were proved for cultivating *S. thermosulfidooxidans*. For the heterotrophic conditions basal mineral media was supplemented with glucose 1.2 mM and yeast extract 0.02%. Mixotrophic conditions were recreated by addition of ferrous iron 70 mM; thiosulfate 2mM; glucose 1.2 mM and yeast extract 0.02% (Karavaiko et al., 2001). Cultures were incubated at 45°C under shaking (120 rpm).

Growth on thiosulfate, tetrathionate and ferrous iron was also evaluated. Tetrathionate and thiosulfate were added at 5 g/l to the basal mineral media supplemented with ferrous iron 5 g/l; ferrous iron (as iron sulphate from acidic stock solution) was added at a final concentration of 20 g/l for evaluating this compound as

sole energy source. Yeast extract was added at 0.02%. Cultures were incubated at 45 °C under shacking at 120 rpm.

5.2. Cell growth measurement.

Cell concentration was determined by direct count using a Thoma counting chamber. The same sample used for cell counting was used for pH measurement.

Iron determination was made according to Skoog & West, 1963, as follows: Solution 1 consisting of ammonium acetate (400 g/l) and glacial acetic acid (500 ml/l). Solution 2 was 1,10-phenantroline (1g/l). Solution 3 composed of hydroxylammonium chloride (200 g/l). Solution 4 was the standard solution prepared with sulfate iron heptahydrate (3.98 g/l) and hydrochloric acid (91 mM), meant for the calibration curve. First, 5 ml of deionized water, 400 µl of the solution 1, 10 µl of sample or standard, 400 µl of solution 2 were mixed together by vortex and the volume completed up to approximately 10 ml with deionized water. The reaction mixture was then incubated for 15 min at room temperature in the dark, and then the absorbance was measured at 492 nm for ferrous iron. After measuring absorbance for the ferrous iron, the same sample was mixed with 200 µl of solution 3 and incubated again for 15 min in the dark; absorbance was measured again at 492 nm to determine total iron.

5.3. Attachment assays and microscopy

5.3.1 Initial Attachment to sulfur and pyrite

Attachment of cells to sulfur and pyrite was followed through time. *S. thermosulfidooxidans* was cultured in Mac basal salt solution with addition of pyrite or sulfur at a concentration of 5 g/l. The media were inoculated with washed cells previously grown on pyrite at an initial concentration of $1x10^7$ cells/ml. Pyrite grains and sulfur coupons were taken every 24 h from the culture to be stained with 4'.6-diamidino-2-phenylidole (DAPI). The DAPI staining is a fluorescent stain which allows visualization of cells by binding to DNA (Morikawa & Yanagida, 1981).

Samples were first taken and rinsed with particle-free water (previously filtered with 0.2 µm filters) in order to remove and to avoid staining of planktonic cell which may be over the surface but not attached and also to remove components of the media which may interfere. Thereafter DAPI (0.2%) was applied all over the surface and samples were stained for 10 min, after staining the excess of DAPI was rinsed out with water, and finally a glycerol solution was added (Citifluor, Ltd. AF2) in order to prevent fading (Bellenberg et al., 2012). Days when cells were already attach to sulfur and pyrite surfaces were chosen for lectin staining.

In the case of sulfur coupons, elemental sulfur was melted and poured over common glass in order to make a flat surface proper for microscopy purposes. These "sulfur coupons" were then autoclaved at 110°C for 30 min. The pyrite used was previously milled to size of 50-100 µm and sterilized by incubation under 180°C for 12 h (Schippers & Sand, 1999).

After microbial growth on media with sulfur and pyrite, confocal laser scaning microscopy (CLSM) of samples stained with DAPI was performed (LSM 510 Carl Zeiss® jena). Images were obtained with the 100 x oil objective and the software LSM 510 version 3.2. From each sample of each day 10 images where obtained.

5.3.2 Lectin staining

Lectins are carbohydrate binding proteins highly specific to a given monosaccharide or simple oligosaccharides. Lectins can be found in plants, microbes and even higher animals. In microscopy studies, labeled lectins with fluorescent substances are very useful tools to visualize samples and determine their polysaccharide components (Slifkin & Doyle, 1990), lectins were used in order to visualize polysaccharides from biofilms (Bellenberg et al., 2012).

According to the initial attachment to sulfur and piryte assays, days where cell agglomeration over reduced spaces was observed, were chosen for staining with lectins. These times were chosen due to the high level of cell population over the

surface and then it is presumed that EPS in these times is already synthesized. Initially samples were stained with DAPI as described before, after they were stained with the lectins (listed in Table 1) for 40 min and finally rinsed with water. Samples were stained in darkness. For staining planktonic cells, $1x10^6$ cells/ml were filtered through polycarbonate filters (0.22 µm) with the help of a vacuum device, and the staining procedure was the same as for pyrite grains and sulfur coupons; lectins were labeled either with TRITC or FITC (Bellenberg et al., 2012).

Table 1: Lectins used in this study.

Lectin/ Abreviation	Target
Concanavalin A (ConA)	α-Manose; α-Glucose
Peanut Agglutinin (PNA)	β-Galactose
Erythrina Cristagalli (ECA)	Galactose; Galactosil (β-1.4);N-Acetylglucosamine
Soybean Agglutinin (SBA)	N-Acetylglucosamine (α and β); galactopyranosyl
Ulex Europeaus agglutinin I (UEAI)	α-1.2 fructose
Pokeweed Miotgen (PWM)	N-Acetylglucosamine
Bandeiraea Simplicifolia isolectin (BSI)	α-D-Galactosyl residues and N-acetyl-α-D-galactosaminyl residues
Phaseolus Vulgaris Agglutinin E (PHAE)	Gal (β1.4) GlcNA (β1.2) or Man (α-1.6)

5.4 *S. thermosulfidooxidans* EPS characterization

5.4.1 EPS extraction

EPS was extracted from pyrite or sulfur grown cells (5 L cultures) after one week of incubation. First, the culture was centrifuged for 12 min at 7500 rpm at 4°C, the

supernatant was collected and it was denoted as “colloidal fraction”. The cell pellet was resuspended to 2 L volume in Mac basal salt solution and centrifuged again, after which the supernatant became the “washed fraction”.

The cell pellet was then resuspended in 120 ml of EPS extraction buffer (50 mM Tris pH 8, 30 mM crown ether, Sigma-Aldrich) and samples were incubated at 4°C for 2 h under shaking (180 rpm).

After incubation with EPS extraction buffer, the sample was centrifuged under the same conditions as before and supernatant was collected. This supernatant was denoted as capsular EPS (first extracted fraction). A second incubation with EPS buffer under the same conditions as the first one was made. The sample was centrifuged again and supernatant was collected obtaining another capsular EPS (second extracted fraction). All fractions were filtered to remove remaining cells (0.2 µm) (Wuertz et al., 2001; Aguilera et al., 2008; Subramanian et al., 2011). Later, EPS fractions were dialyzed first for 12 h in running water and later in 5 l volume for 96 h (changing the water every 24h). Finally, aliquots of all EPS fractions extracted were lyophilized in order to obtain the dry weight of each fraction and aliquots were taken for chemical analyses.

5.4.2 Spectrophotometry.

Nucleic acids, proteins, humic acids and polysaccharides concentration in the EPS was determined by spectrophotometry after EPS extraction.

Nucleic acids were determined according to Burton, 1955 as follows: diphenylamine solution (diphenylamine 99%; sulfuric acid 95%; acetic acid 99%); acetaldehyde solution (acetaldehyde 21%); perchloric acid solution (0.5 N). The reaction mix contained 200 µl of sample, 200 µl of perchloric acid solution and 800 µl of the diphenylamine solution mixed with the acetaldehyde solution (1:200). Samples were incubated at 30°C for 16 to 20 h; finally absorbance was measured at a wavelength of

600 nm. Salmon sperm DNA was used as standard, stock solution and dilutions were made in perchloric acid (0.5 N).

Polysaccharides were determined according to Dubois et al., 1956 with aqueous 5 % (w/v) phenol and sulfuric acid 95 % at a wavelength of 492 nm with glucose as standard, the reaction time was 10 min at room temperature, the reaction mixtures were composed of 1 ml of sulfuric acid, 0.2 ml of phenol and 0.2 ml of sample.

Protein determination was made according to Lowry et al., 1951. The Lowry reagent was prepared mixing three solutions: NaOH (0.143 N) and Na_2CO3 (0.135 N) (solution A); $CuSO_4$ (0.057 M) (Solution B), and sodium tartrate (0.124 M) (solution C). All the solutions (A, B and C) were mixed at a ratio of 100:1:1 to obtain the Lowry reagent. A volume of 0.5 ml of sample was mixed with 0.7 ml of Lowry reagent and incubated for 20 min at room temperature in the dark and then mixed with 0.1 ml of diluted foulin reagent (5:6 Foulin 2 N:water) and incubated again at room temperature for 20 min. Absorbance was measured at 750 nm and Bovine Serum Albumin (BSA) was used as standard.

The determination of Humic acids was made as the protein determination but replacing sodium tartrate for water (Frolund et al., 1996). Proper corrections were applied in order to obtain normalized absorbances.

In the cases where no signal was detected from any of the measurements made, liophylized aliquots of these samples were resuspended in PBS buffer in a lower volume from the initial volume liophilized (10 times more concentrated than initially) in order to increase the chance of getting a measurable signal from the sample.

5.4.3 Determination of cell lysis.

To determine the level of cell lysis the enzyme glucose 6-phosphate dehydrogenase (G6PDH) was measured (Flemming & Wingender, 2010) as follows: buffer Tris HCl (50mM), D-glucose-6-phosphate (2 mM), NADP (1mM), $MgCl_2$ (10 mM) and 100 µl of EPS sample at a final volume reaction of 1 ml. The reaction mixture was incubated

at 37°C for 60 min. Absorbance values were measured at 340 nm every 15 min during the whole incubation (NG & Dawes, 1973). In order to show results in terms of percentage, aliquots from the culture (10 ml) of the medium were retrieved before extraction and cells were lysed by sonication (5 intervals of 7 min each with 3 min breaks between intervals at 40 W, samples were kept on ice through the whole process). G6PDH was measured, becoming the positive control and the reference point for comparison and establishment of cell lysis percentage for the EPS samples.

Additionally the whole spectra (wavelengths from 190 nm to 900 nm) of EPS samples were measured in order to have an overview on the compounds which are in the unknown percentage.

5.5 Molecular Biology techniques

5.5.1 RNA isolation

The acid phenol RNA extraction method was used. First, cells cultured on sulfur and pyrite (1,2 x 10^8 cells/ml) were resuspended on 700 µl of lysis solution (sodium acetate 20 mM pH 5.5, SDS 1% and EDTA 2mM) previously warmed up for 10 min at 65°C. Samples were incubated for 10 min at 65°C, mixing every minute for 15 s, then one volume of acid phenol was added and samples were incubated for 10 min and centrifuged at 10.000 x g for 7 min. Supernatants were recovered and mixed with one volume of acid phenol. A second centrifugation under the same conditions as before was done and one volume of phenol:chloroform:isoamilic alcohol (25:24:1) was added. Supernatants were recovered and mixed for 3 min with an equal volume of chloroform. Samples were then centrifuged and supernatants recovered. Nucleic acids were precipitated by addition of 1 ml of isopropanol (Vera et al., 2009).

RNA capture column provided by Roche® was used (High Pure RNA Isolation Kit). RNA cleaning from DNA was done with DNAse I (Roche) free of RNAse, 30 U of enzyme at 25°C for 20 min with $MgCl_2$ 5 mM (Vera et al., 2009). Absorbances from samples at 230, 260 and 280 nm were measured in order to determine the

concentration of nucleic acids and proteins; also an electrophoresis gel was run to determine RNA integrity.

5.5.2 DNA Isolation

DNA was extracted as described by Aljanabi & Martinez, 1997, initially cultures were centrifuged (7500 rpm for 12 min) and cell pellets were collected and resuspended in 1 ml of Mac basal salt medium. Samples were centrifuged again under the same conditions and cell pellets were resuspended in 400 µl of salt buffer containing 0.4 M NaCl, 10 mM Tris-HCl pH 8 and 2 mM EDTA. Then 40 µl of 20% SDS were added to lyse the cells and samples were incubated at 55°-65°C for 1 h, then 300 µl of 6 M NaCl solution were added and samples were vortexed and centrifuged at 11000 rpm for 30 min. After centrifugation, supernatants were collected and mixed with an equal volume of isopropanol and incubated for 1 h at -20°C. Then, samples were centrifuged for 20 min at maximum speed at 4°C, pellets were washed with 70 % ethanol, then dried and finally resuspended in 100 µl of nanopure water.

5.6 Bioinformatics search of genes potentially involved in the biofilm forming process.

5.6.1 Sequences search in the *S. thermosulfidooxidans* genome

The genome information of *Sulfobacillus thermosulfidooxidans* DSM 9293 was obtained from The Genome Portal of the Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/) during May and June 2012. Sequences related to transport of polysaccharides by bioinformatic analysis, cluster of orthologus groups (COG), motif analysis, KEGG pathways (Kyoto Encyclopedia of Genes and Genomes http://www.genome.jp/kegg/), were obtained directly. Sequences previously described to have a key role in biofilm formation (Cuthbertson et al., 2010; Whitfield, 2006; Bomchil et al., 2003; Donlan, 2002; Branda et al., 2006; Matsukawa & Greenberg, 2004; Yildiz & Schoolnik, 1999) were obtained from the

National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). With these sequences previously described to be involved in the biofilm formation process a BLAST (Altschul et al., 1990) against *S. thermosulfidooxidans* genome was run and sections of the genome which aligned to the reference sequences were subtracted from the whole genome and BLAST (http://blast.ncbi.nlm.nih.gov/) searches were run with them against the whole NCBI nucleotide collection database.

5.6.2 Primer design for sequences potentially involved in EPS synthesis and exportation.

PCR primers were designed using the European Molecular Biology Open Software Suite (EMBOSS) with the algorithm Primer 3 (Rozen & Skaletsky, 2000). In order to have a better and summarized overview of the structure and composition of the locus, the genes potentially related to the EPS production and exportation results of the bioinformatics search are schematically shown with the help of ARTEMIS (http://www.sanger.ac.uk/).

According to the results of the bioinformatics analysis the genes Sulth_1631, Sulth_1632 and Sulth_1635 were chosen, and primers for PCR amplification were designed with the algorithm Primer 3 in the EMBOSS interface

6. Results

6.1. Growth kinetics on Mac basalt salt medium.

S. thermosulfidooxidans was grown in the presence of different energy substrates, iron, thiosulfate and tetrathionate. Cultures under heterotrophic conditions, in presence of glucose, and mixotrophic conditions were also employed. Thiosulfate and tetrathionate were also used as sole source of energy, without ferrous iron; however, the growth was not as high as expected (data not shown). Cultures were made by triplicate.

Among the energy sources tested, the iron was the one with higher cell concentration. On the other hand, cells grown on thiosulfate and tetrathionate reached similar cell concentrations but the behavior of the cell concentration was different in these cultures. Cells grown on iron, at day 9^{th}, reached cell populations of 4.3 x 10^8 cells/ml, and 3.8 x 10^7 cells/ml in the case of thiosulfate around six days, with tetrathionate in the same day population reached 2 x 10^7 cells/ml. For the mixotrophic and heterotrophic conditions, the cell concentrations through time were below the medium with iron as energy source. Mixotrophic conditions had higher cell concentrations, a maximum of $1x10^8$ cells/ml around the third day of culture, than cells cultured under heterotrophic conditions, $1.8x10^7$ cells/ml around the same day (Fig 5).

In the case of the growth under heterotrophic conditions, there was not growth at all when compared to mixotrophic conditions; the cell concentration was constantly ranging around values of 10^7 cells/ml during the whole culture. In the culture under heterotrophic conditions, the pH was also constantly ranging around values of 2.3. For the case of the culture under mixotrophic conditions, the cell concentration increased from the very first day until reaching higher values after three days; after that the concentration started to decrease. The pH on the medium under mixotrophic conditions also remained constant during the whole culture ranging around values of 1.5 and slightly decreasing by the end of the culture.

In the medium supplemented with tetrathionate the cell concentration increased up to the day three and decreased by the day five. From the fifth day up to the sixth day the population remained at close values around $2x10^7$ cells/ml and finally decreased on the day seven. The pH on the medium with tetrathionate decreased on the early stages and remained at low values during the whole culture. The medium with thiosulfate showed a long adaptation phase of around five days and a half and cell concentration increased up to six days and a half; after cell concentration decreased. The pH on the medium with thiosulfate also decreased during the first stages of the culture and remained under low values during the whole culture.

During the first stages of cells cultured on iron, an adaptation took place up to the second day. From the second day on the cell concentration increased up to day seven, slightly decreased on day eight, increased again in the day nine and after that decreased. Finally, in the medium with iron, pH values also dropped in the beginning down to 2.5 within 36h and remained almost constant all over the incubation. The concentration of ferrous and total iron dropped in the beginning to 0.033 and 2.2 mg/l, respectively, and remained constant around these values also for the rest of the cultivation.

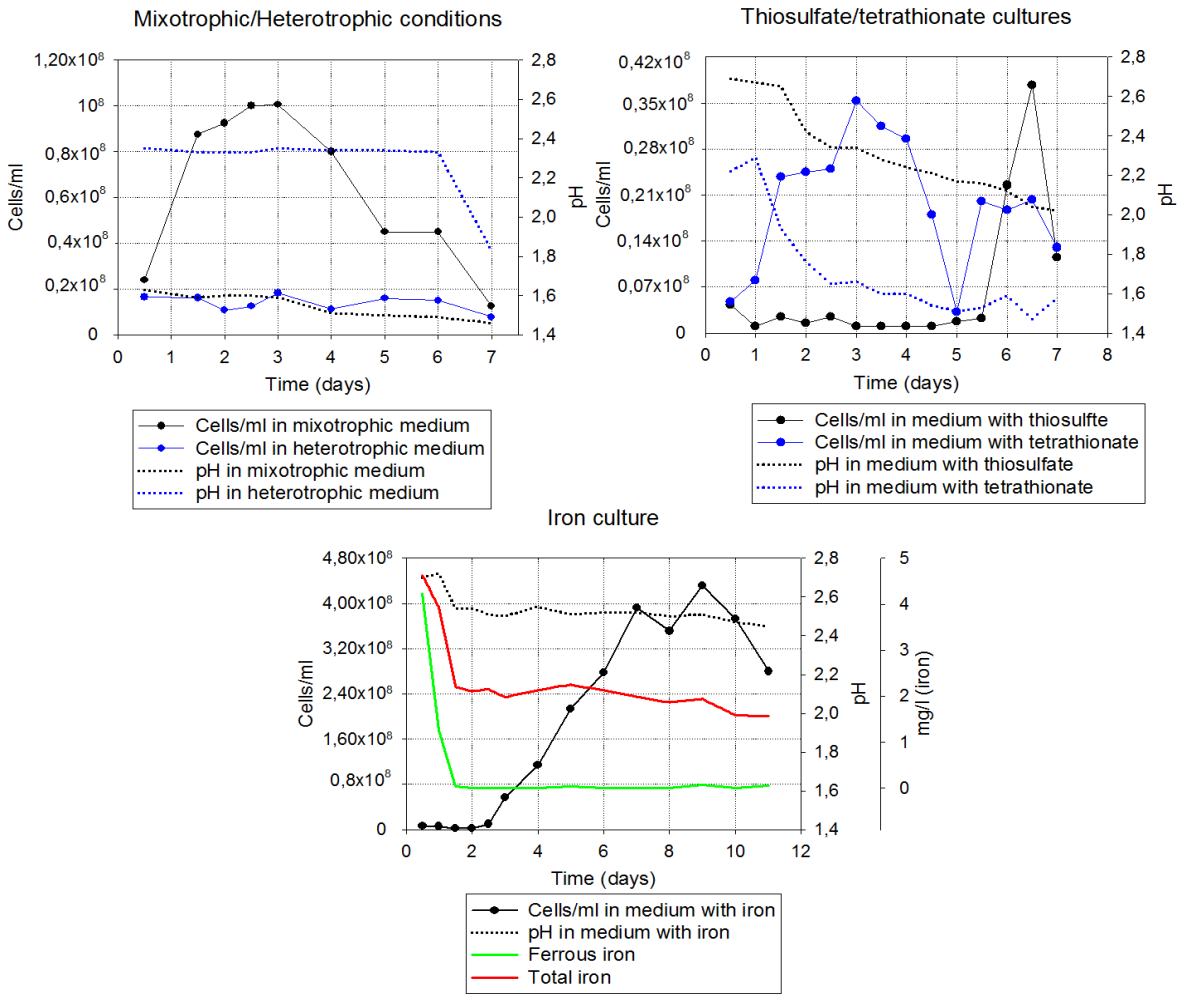


Figure 5: Growth kinetics of *S. thermosulfidooxidans* on different substrates, cell concentration (cells/ml) and pH were measured for all samples, in the case of medium supplemented with iron, ferric and total iron concentration was measured.

6.2. Growth kinetics in DSMZ basal salt medium.

Basal salt medium DSMZ for *Acidithiobacillus ferroxidans* (© 2007 DSMZ GmbH – all rights reserved) was also employed. The DSMZ medium was supplemented also with different energy sources, such as iron sulphate, tetrathionate and thiosulfate with iron sulphate. However, growth achieved with this basal salt medium was not as high as with Mac, and in general terms for iron, tetrathionate and thiosulfate cell concentration was below 100 and 1000 times compared to Mac grown cells (data not shown).

6.3. Attachment assays and microscopy

6.3.1. Initial attachment to sulfur and pyrite

In the initial attachment to piryte and sulfur it can be observed that the aggregation of cells on reduced surface spaces through time was different. In the case of sulfur, cell aggregation was observed in the day 8; on the other hand the cell aggregation over piryte was observed from even the first day. Fig. 6 shows the days where major changes on colonization level over piryte and sulfur were observed during cultivation of *S. thermosulfidooxidans* over these surfaces. In sulfur grown cells it was observed that cell population on the surface increased in days 3 and 4, while cell aggregation was seen from day 8 and after by day 10 it seems to be a cell disruption from the surface (Fig. 6), the attachment was followed up to the 11^{th} day.

In the case of the pyrite, cell population over the surface was higher compared to sulfur surfaces; cells agglomeration over reduced spaces on the pyrite was observed since the first day. This colonization level over the piryte, remained constant over all the following days. Although in some days the cell population over the surface was higher as in the case of the day 4, for example (Fig. 6). For detailed following on the attachment to piryte and sulfur during the eleven days, please refer to appendix 9.1 and 9.2.

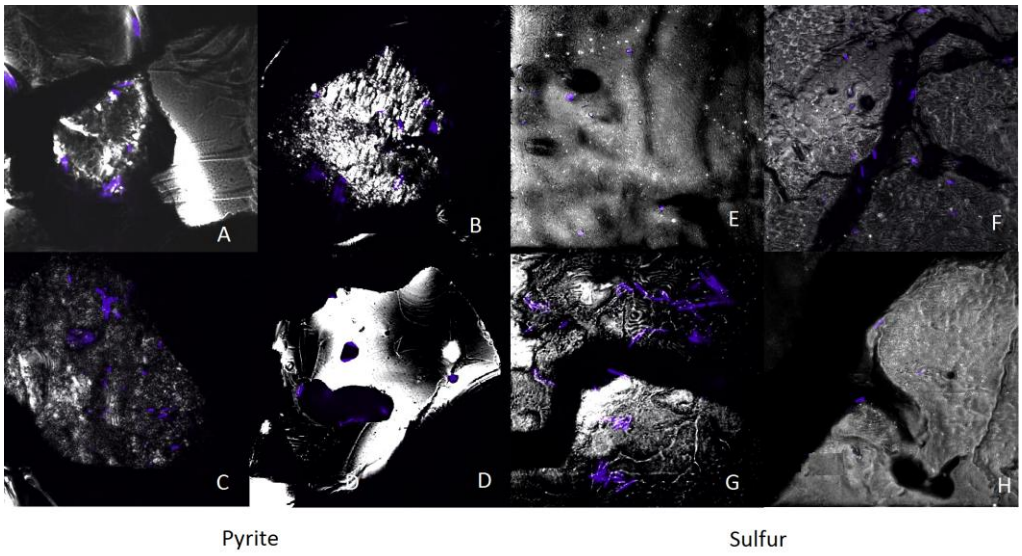


Figure 6: Initial attachment of *S.thermosulfidooxidans* cells to sulfur or pyrite through time. 364 nm for DAPI, 543 nm for reflection channel. A to D correspond to cells grown on pyrite, A: day 1, B: day 4, C: day 5, D:day 8; E to H correspond to cells grown in sulfur, E: day 1, F: day 4, G: day 8, H: day 10.

6.3.2. Lectin staining.

Several lectins (Table 1 in materials and methods) were used in order to visualize some of the components present in the EPS produced by *S.thermosulfidooxidans*. The lectin staining was done when the initial attachment to sulfur and pyrite assays occurred, as described previously in materials and methods.

Only ConA produced a visible signal under all conditions tested; while PNA and SBA gave positive signals only in the sulfur grown cells (planktonic state); signals from the lectin PHAE were also detected in the medium with sulfur but only for cells in sessile state.

In the case of pyrite grown cells, signals from the lectins UEAI and PHAE were detected for planktonic state (Fig. 7). The results of all the lectin stainings are summarized in Table 2. Images of staining with all the lectins including both sessile and planktonic can be found in the appendix 9.3 and 9.4.

The results suggest production of α-manose, α-glucose, galactose β 1-4 and N-acetylglucosamine from sessile cells grown in sulfur. On the other hand, for sessile cells grown on pyrite only α-manose and α-glucose were detected. Apart from α-

manose and α-glucose, α and β acetylgalactosamine and galactopyranosyl were also detected in planktonic cells grown in sulfur. For planktonic cells grown in pyrite α-manose, α-glucose, α 1-2 fructose, N-acetylglucosamine, galactose β 1-4 and manose α 1-6 were detected.

Table 2: Results of lectin staining for planktonic and sessile cells of *S.thermosulfidooxidan* grown on piryte and sulfur.

	Sulfur		Pyrite	
Lectin	**Sessile**	**Planktonic**	**Sessile**	**Planktonic**
ConA	+	+	+	+
PNA	-	+	-	-
ECA	-	-	-	-
SBA	-	+	-	-
UEA I	-	-	-	+
PWM	-	-	-	-
BSI	-	-	-	-
PHAE	+	-	-	+

+: positive signal observed under the microscope; -: no positive signal observed under the microscope

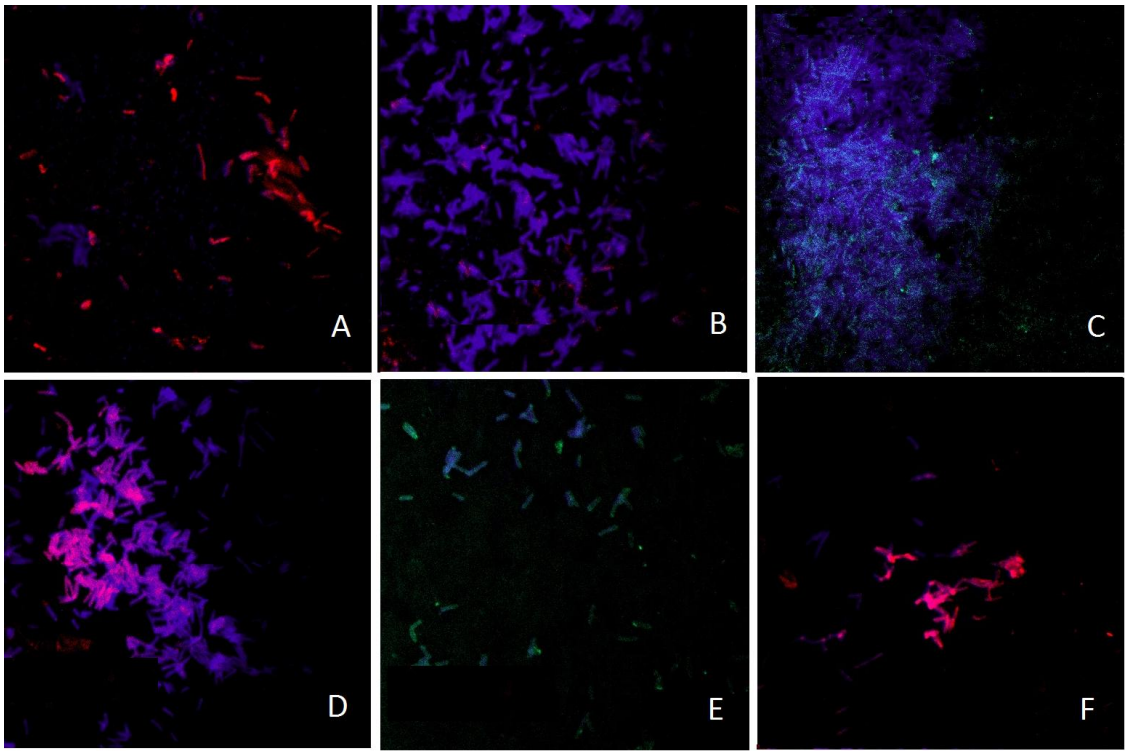


Figure 7: *S. thermosulfidooxidans* cells stained with different lectins. Excitations: 364 nm for DAPI (blue), FITC 490 nm (green), TRITC 557 nm (red). From A to C cells grown on pyrite are shown. A: Con A, B: PHAE, C: UEAI; from D to F cells grown on sulfur are shown, D: PNA, E: SBA, F: PHAE.

6.4. *S. thermosulfidooxidans* EPS analysis.

The EPS produced by cells grown in pyrite and sulfur cultured for one week was extracted and analyzed. Humic acids, polysaccharides and proteins were found, nucleic acids were not detected in the samples.

EPS from ferrous iron grown cells and from planktonic cells cultured in sulfur was extracted. Nevertheless dry weights were around 3 mg and even no detectable in some of the fractions. In contrast, 30 mg were recovered from the sessile cells grown in pyrite or sulfur. The amounts of EPS produced by planktonic cells were not enough to be analyzed. Therefore, the following results correspond to the EPS extracted from pyrite and sulfur grown sessile cells.

6.4.1. EPS from pyrite grown cells.

The main proportion of the dry weight (more than 60% and up to 93% in some cases) was unknown components of the EPS. The remaining percentage was distributed within the measured compounds in proteins, humic acids and polysaccharides.

For all the EPS fractions extracted from pyrite grown cells, the humic acids concentration was the highest with the only exception of the capsular fraction (second extraction) in which the concentration of humic acids was as high as the concentration of polysaccharides (14.2 %) (Fig. 8). The concentration of humic acids for the capsular fraction (first extraction) was 20.7%. For the rest of the fractions the concentration of humic acids was 4% and 5.6% in the washed fraction and colloidal fraction respectively. The concentration of polysaccharides in the capsular first extraction was 1.8%; for the washed fraction was 0.08% and for the colloidal fraction was 1.3%. The concentration of proteins in the capsular second extraction was 10.3%; for the capsular first extraction was 11.2%; for the washed fraction was 2.5% and finally 3.4% for the colloidal fraction.

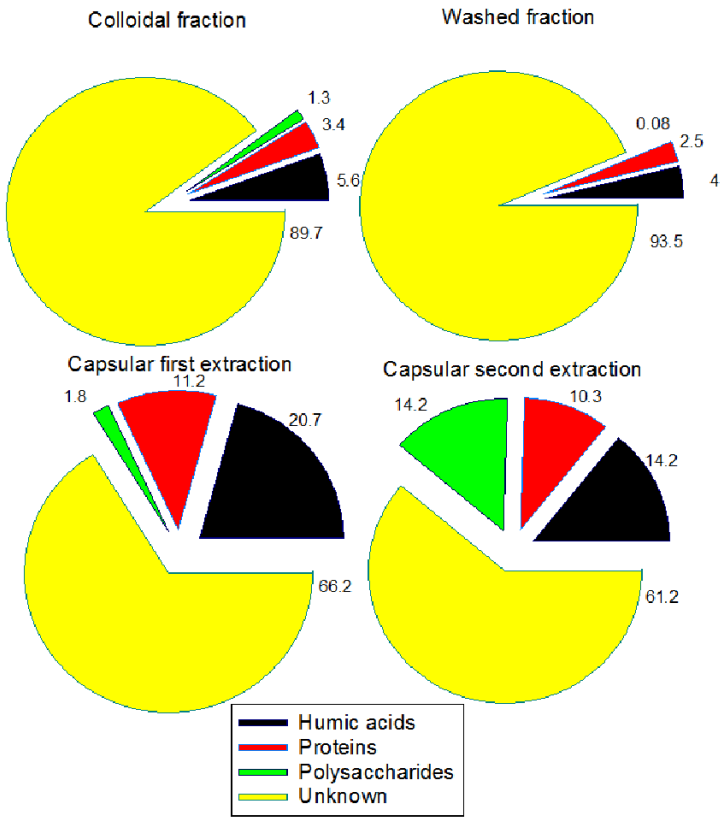


Figure 8: Percentage of compounds measured in the EPS from pyrite grown *S. thermosulfidooxidans* cells.

6.4.2. EPS from sulfur grown cells.

As in the case of pyrite grown cells, the unknown components of the EPS were also the largest among all the fractions (more than 80% and up to 91% of the dry weight). The remaining percentage was distributed among the measured compounds: humic acids, proteins and polysaccharides.

EPS extracted from sulfur grown cells, showed more humic acids than proteins and the polysaccharides in the lowest proportion of the EPS (Fig. 9). The highest humic acids concentration was 134.9 µg/mg of dry weight of EPS corresponding to the capsular fraction (first extraction) and 53.6 µg/mg for the washed fraction being the lowest (Fig. 9). The highest protein concentration was in the capsular first extraction being as high as 6.7 % and on the other hand, 3.5 % was the lowest corresponding to

the washed fraction. Polysaccharides were detected in the colloidal and the washed fractions (0.5 and 0.2 %).

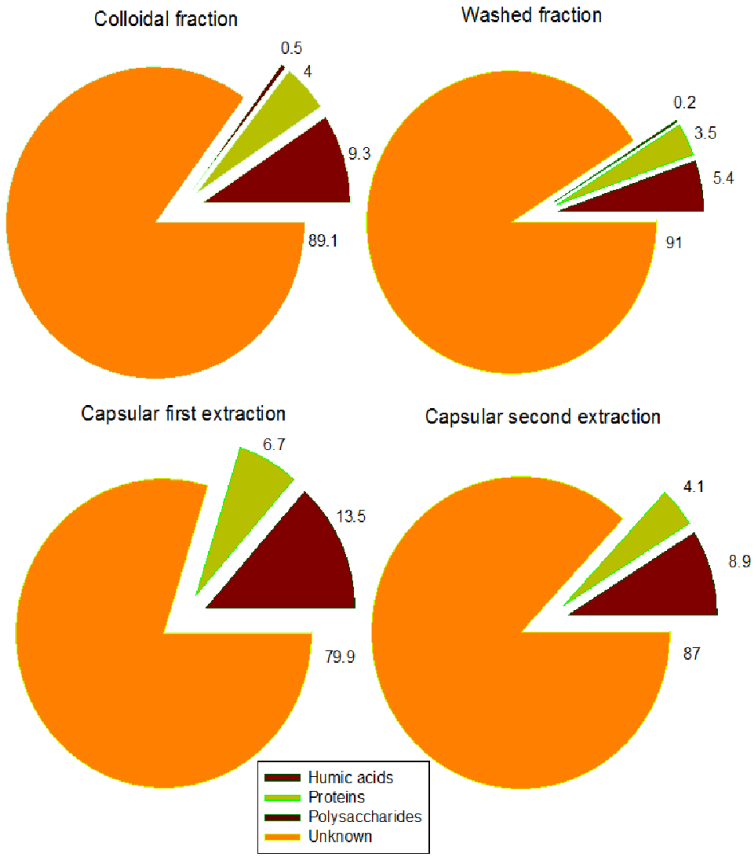


Figure 9: Percentage of compounds measured in the EPS from sulfur *S. thermosulfidooxidans* cells.

The data are also shown in the comparison table 3, where the concentrations for each compound measured can be observed together with the values of the negative controls. Negative controls were obtained by making extraction of cultures which were not inoculated. It is thought that by the end of the EPS extraction process the interference should be higher because the strongest signals within the negative controls were achieved for the capsular second extraction (data not shown). For this reason, the negative control was measured with the capsular second extraction. Standard deviation values and unknown percentage of compounds are also shown for each measurement.

Table 3: concentration of the different compounds obtained from the spectofotometric measurements.

Energy source	Fraction	HA	SD	PRO	SD	POL	SD	NA	UK
EPS from pyrite grown cells	**Colloidal fraction**	31,12	0,0131	18,91	0,0000	7,05	0,0344	NDT	89,70%
	Washed fraction	19,89	0,0025	12,73	0,0000	0,42	0,0442	NDT	93,50%
	Capsular first extraction	131,20	0,0012	70,85	0,0006	11,92	0,0833	NDT	66,20%
	Capsular second extraction	25,94	0,0045	18,73	0,0010	3,08	0,0064	NDT	61,20%
	Negative control	6,99	0,0015	0,00	0,0000	0,00	0,0040	NDT	ND
EPS from sulfur grown cells	**Colloidal fraction**	58,30	0,0095	31,09	0,0066	3,26	0,0096	NDT	89,10%
	Washed fraction	32,00	0,0068	20,73	0,0017	1,19	0,0232	NDT	91%
	Capsular first extraction	41,01	0,0253	20,30	0,0032	0,00	0,0061	NDT	79,90%
	Capsular second extraction	26,63	0,0055	12,36	0,0036	0,00	0,0058	NDT	87%
	Negative control	7,68	0,0036	0,00	0,0000	0,00	0,0040	NDT	ND

HA: humic acids; SD: standard deviation; PRO: proteins; POL: polysaccharides; NA: nucleic acids; UK: unknown compounds; NDT: not detected; ND: not determined. n=3.

6.4.3. Cell lysis verification

To assure that the measurement of compounds in the EPS samples analyzed were not due to intracellular content, the percentage of cell lysis was determined. The concentration of planktonic cells on sulfur medium before extraction was $8.7x10^7$ cells/ml and $4.25x10^7$ cell/ml for the medium with pyrite.

The results of the cell lysis measurements were low and in some cases under the detection limit of the technique (washed fraction and the capsular second extraction) results are shown in Fig. 10. The highest cell lysis occurred in the capsular first extraction from pyrite grown cells, where it was estimated as 0.04 % from the initial cell concentration at the beginning of the extraction. In the case of the EPS extracted from sulfur grown cells, the highest percentage of cell lysis was estimated as 0.08 % from the initial cell concentration before EPS extraction. In Fig. 10 the percentages of cell lysis according to G6PDH activity are shown, absorbance values at 60 min and

15 min were deducted and these absorbance values were used to determine cell lysis percentage for each fraction.

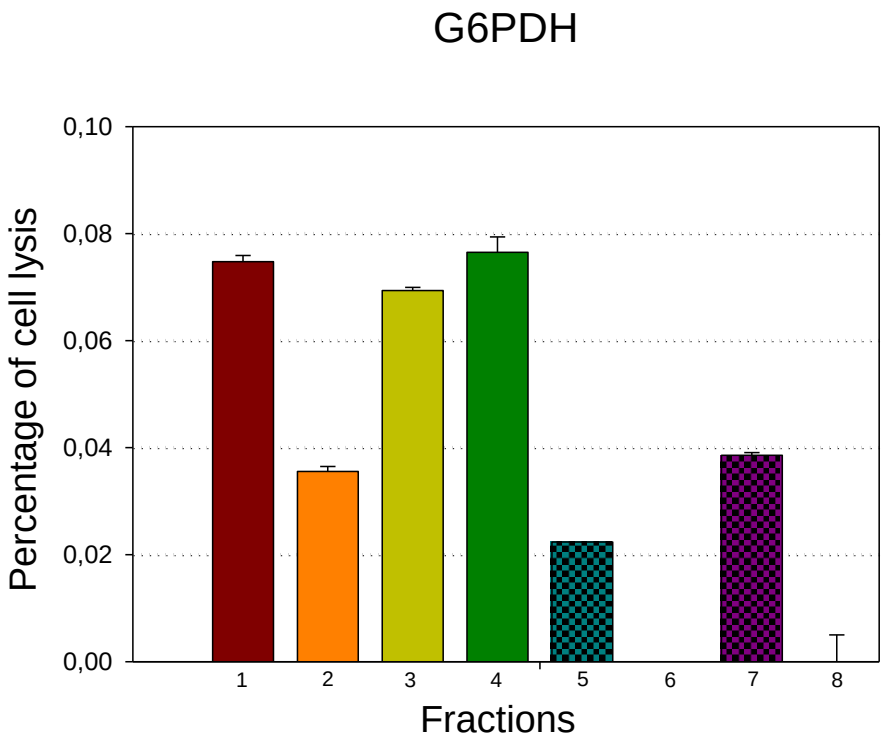


Figure 10: Cell lysis control for all the fractions, Glucose-6-phosphate-dehidrogenase. n=3, 1: Colloidal fraction sulfur; 2: Washed fraction sulfur; 3: Capsular first extraction sulfur; 4: Capsular second extraction sulfur; 5: Colloidal fraction pyrite; 6: Washed fraction pyrite; 7: Capsular first extraction pyrite; 8: Capsular second extraction pyrite.

According to the measurements, DNA concentration in all the samples was lower than the detection limit of the technique. The signal for DNA was undetectable even from the lyophilized fractions which were tenfold concentrated.

6.4.4. Spectra analysis.

Absorbance was measured in the entire light spectra (190 nm to 900 nm) for all the samples. It could be observed that the highest absorbance values are present in all the fractions at around 281 and 293 nm, all the absorbance values were below the negative control. The results suggest the presence of aliphatic compounds of low

complexity (Harris, 1999). The results are shown in Fig. 11. The whole spectra can be found in appendix 9.5.

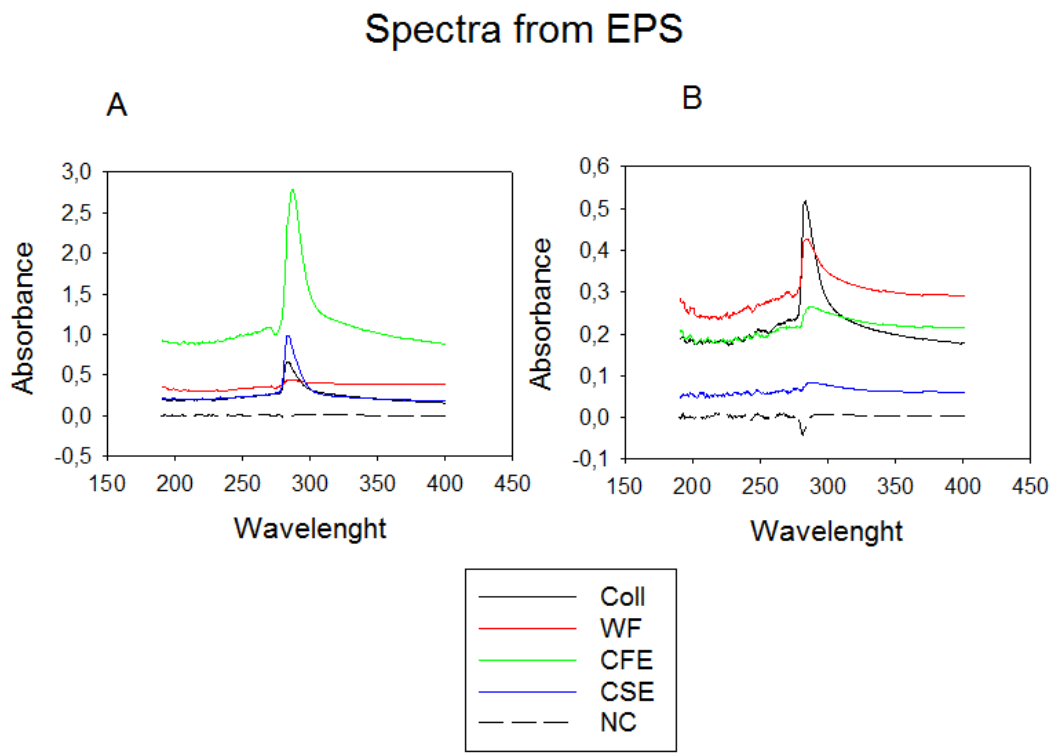


Figure 11: Measurement of the whole spectra from the samples of EPS. A: EPS samples from piryte grown cells; B: EPS samples from sulfur grown cells. Coll: colloidal fraction; WF: washed fraction; CFE: capsular first extraction; CSE: capsular second extraction; NC: negative control.

6.5. Bioinformatics

Blast searches were done with reference sequences previously reported to be involved in the process of EPS production and biofilm formation (Cuthbertson et al., 2010; Whitfield, 2006; Bomchil et al., 2003; Donlan, 2002; Branda et al., 2006; Matsukawa & Greenberg, 2004; Yildiz & Schoolnik, 1999). Blast results showed that those sequences are distributed along the genome of *S.thermosulfidooxidans* and have matches with different percentage of identity (from 20 % to 100 %) (see appendix 9.6). Nevertheless, the sequence coverage, in many cases, was not high enough to be considered as a positive match; the expectation values were also in some cases even above zero for some 100% identity matches. Therefore, none of these sequences were

considered as a positive match. Sequence alignment between these reference sequences indicated that the level of similarity is high among them, even 90 % in some cases (data not shown).

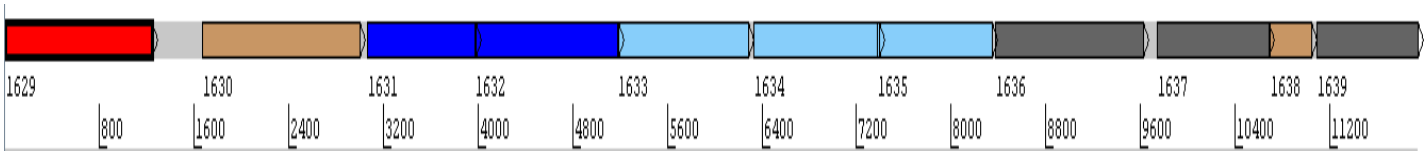
It has been reporte that EPS production and exportation may occur by three mechanisms: Wzx/Wzy dependent pathway, ABC transporter dependent pathway and synthase dependent pathway (Cuthbertson et al., 2010). These sequences were searched over the genome of *S. thermosulfidooxidans* DSM 9293 using different databases. According to the search made in this thesis, the genome of *S. thermosulfidooxidans* DSM 9293 posses several sequences encoding for ABC transporters proteins and Polysaccharide transporters, two families of interest in the present study due its relation with EPS production and exportation as it has been explained before. Some of them are Sulth_1174, Sulth_1175, Sulth_1176, Sulth_1266, Sulth_1267, Sulth_1631 and Sulth_1632 (Table 4). Those sequences might be good candidates for studies of gene expression levels (Fig 12).

Table 4: Genes potentially involved in EPS biosynthesis in *S. thermosulfidooxidans* genome.

Locus	Sulth_0304	Sulth_0308	Sulth_0309	Sulth_0478	Sulth_0506	Sulth_0583	Sulth_0584
COG	+	+	+	+	+	+	+
KO		+	+			+	+
PCG_CTP	+	+	+			+	+
KMT							
PCGFP		+	+			+	+
Locus	Sulth_0585	Sulth_0779	Sulth_1070	Sulth_1174	Sulth_1175	Sulth_1176	Sulth_1265
COG	+	+	+	+	+	+	+
KO	+			+	+	+	+
PCG_CTP				+	+	+	
KMT				+	+	+	+
PCGFP			+		+	+	+
Locus	Sulth_1266	Sulth_1267	Sulth_1446	Sulth_1447	Sulth_1448	Sulth_1600	Sulth_1631
COG	+	+	+	+	+	+	+
KO	+	+	+	+	+		+
PCG_CTP	+	+	+	+	+		+
KMT	+	+					
PCGFP	+			+	+		

Locus	Sulth_1632	Sulth_1781	Sulth_1782	Sulth_2763	Sulth_3761	Sulth_3762	Sulth_3763
COG	+	+	+	+	+	+	+
KO	+	+	+	+	+	+	+
PCG_CTP		+				+	+
KMT	+	+		+			
PCGFP		+	+	+		+	+

COG: cluster of orthologous groups related to transport and metabolism of carbohydrates; KO: KEGG orthologie with filtering by "transport"; PCG_CTP: Protein coding genes coding transmembrane proteins; KMT: Protein coding genes connected to KEGG pathways-membrane transport pathway; PCGFP: Proteins coding genes with function prediction with "transport" as filtering.



Loci	Start	End	Match
1629	1	1235	Transposase
1630	1669	2993	Hypothetical protein
1631	3065	3973	ABC-2 type transporter
1632	3974	5182	Teichoic acid-transporting ATPase
1633	5183	6274	Glycosiltransferase
1634	6331	7389	Glycosiltransferase
1635	7376	8335	Glycosiltransferase
1636	8378	9616	Helix-turn-helix domain
1637	9744	10682	Beta-lactamase
1638	10698	11036	Hypothetical protein
1639	11093	11936	dTDP-4-dehydrorhamnose reductase

Figure 12: Locus of the genes considered good candidates for RT-PCR.

6.5.1. Primer design for sequences potentially involved in EPS synthesis and exportation.

According to the results of the bioinformatics analysis the genes Sulth_1631, Sulth_1632 and Sulth_1635 were chosen, and primers for PCR amplification were designed with the algorithm Primer 3 in the EMBOSS interface (Table 5).

Table 5: sequences selection and results of primer design with the algorithm primer 3.

Locus	Orientation	Lenght	Tm (°C)	MW (g/mol)	GC-content	Sequence
1631	Forward	20	55,3	6135	45%	tgggccttattcttttggtg
	Reverse	20	55,3	6100	45%	gcggttcaatccgtctaaaa
1632	Forward	20	55,3	6141	45%	gtcatgcaaaaatcgggtct
	Reverse	20	57,3	6045	50%	cctaaacgcatcaggtccat
1635	Forward	20	55,3	6135	45%	tgggccttattcttttggtg
	Reverse	20	55,3	6100	45%	gcggttcaatccgtctaaaa

Tm: temperature of melting; MW: molecular weight.

Unfortunately no successful PCR amplification with genomic DNA was obtained (data not shown), for this reason it was decided not to perform any RT-PCR.

7. Discussion

7.1. Growth kinetics

Differences in the growth of *S. thermosulfidooxidans* were observed with different energy sources. This is an expected behavior since the different conditions in the media have different effect on this microorganism. Although *S. thermosulfidooxidans* is able to growth using sulfur and iron as energy substrates (Ding et al., 2007), it was observed that its growth is higher on presence of iron in comparison of sulfur compounds as it has been reported (Egorova et al., 2004).

In the case of the growth on thiosulfate with ferrous iron, there was no growth at all and only by the 5^{th} day the cell concentration increased. This could be interpreted as a long adaptation phase after which there was some growth. *S.thermosulfidooxidans* can present long adaptation phases that can take even days (Buleav et al., 2011). The growth on tetrathionate with ferrous iron was higher than in thiosulfate with ferrous iron. No growth was achieved under the presence of tetrathionate and thiosulfate without ferrous iron. The degradation of tetrathionate and thiosulfate by *S. thermosulfidooxidans* is influenced by the inoculums used for starting a new culture. Depending on the energy source used to grow the inoculums, the new culture may change its yield in terms of cell concentration (Egorova et al., 2004). Then, it is thought that with inoculums from different energy sources, these results may change. Nevertheless, adaptation on medium with tetrathionate and thiosulfate as sole source of energy was attempted before performing the measurement but no successful growth was achieved. It is known that for *S. thermosulfidooxidans* the number of transfers over the same media can lead to poor or even none growth (Buleav et al., 2011; Muravyov et al., 2010).

It was observed that the growth of *S. thermosulfidooxidans* is higher under mixotrophic conditions in comparison to heterotrophic conditions as it has been reported (Karavaiko et al., 2001). Furthermore the growth of *S. thermosulfidooxidans* can be limited by the concentration of glucose in the medium (Clark & Norris, 1996).

This is due to the fact that enzyme activity changes according to the conditions. Under mixotrophic conditions some enzymes related to carbohydrate metabolism show higher activity compared to heterotrophic and autotrophic conditions (Karavaiko et al., 2001). Furthermore, it is known that depending on the energy source some metabolic pathways can be expressed or repressed. When *S. thermosulfidooxidans* is grown in the presence of pyrite, some of the enzymes related to the TCA cycle are not produced, while in the presence of glucose some of the enzymes related to the Calvin cycle are synthesized (Karavaiko et al., 2002), probably causing differences in the growth.

The medium used for heterotrophic conditions was the only one lacking of iron among all the media used for growing *S. thermosulfidooxidans*. This was the medium with lower growth achieved by *S. thermosulfidooxidans*, therefore it might be thought that this energy source is of great importance for this microorganism.

7.2. Initial attachment to sulfur and pyrite.

The differences observed in the determination of EPS composition can be correlated with the differences observed on the initial attachment of cells to the different substrates. The cell attachment was followed for several days; the cell attachment to pyrite seemed to be faster than cell attachment to sulfur. These different moments of attachment are probably due to the time that the cells need to synthesize the initial matrix for adhesion to the surface. Thereafter, cells are tightly bound due to the increased synthesis EPS and this synthesis is defined by the substrate (Li & Yang, 2007).

EPS production which allows adhesion, is an energy consuming process, therefore the cell needs a source of energy (Kreft & Wimpenny, 2001). In the case of *S. thermosulfidooxidans* it has been observed that it has a higher affinity for iron as energy source than to sulfur (Egorova et al., 2004). Therefore, it is expected that its growth would be higher in medium with iron, as it was observed in the growth kinetics, furthermore it might be the source of more energy for EPS production. If

there is more EPS production in the medium with ferrous iron, it is expected that more cells would be attached to the surface in comparison to a medium without ferrous iron. Although *S. thermosulfidooxidans* is able to grow on sulfur also, consequently it was expected its growth would be higher in pyrite because it contains both sulfur and iron.

Several fields under the microscope were analyzed, and the distribution and adhesion of the microorganisms to the surface does not seem to be a random process, it is ruled by imperfections or scratches over the surface as well as by several biological process (Sand et al., 1995). Therefore, this work should be considered as a preliminary approach to study how *S. thermosulfidooxidans* attaches to sulfur and pyrite surfaces.

7.3. Lectin staining.

As it was observed, the results of the staining with different lectins showed that some cells produce EPS with a different composition in the planktonic state and cells in the sessile state. This could be explained as a response from the cells to the contact with the surface. It seems that only the soluble compounds in the media influence the production of EPS on planktonic state. At later stages, when cells are attached, it is influenced by the surface itself and the components of the media, thus the EPS composition might change. Similar behavior has been reported for *Acidithiobacillus ferrooxidans* cultures, where ferrous iron grown cells do not show interaction with ConA and when cultured in pyrite, there is a clear interaction (Bellenberg et al., 2012).

The biofilm develops in different stages and for achieving and building the final mature structure different mechanisms are involved. One of these mechanisms is the synthesis of adhesins, and other components of the EPS. In planktonic state and early stages of biofilm formation a low amount of EPS is produced and at later stages the production of the adhesins and the rest of EPS components is specific according to the surface (Karatan & Watnick, 2009). This is also an explanation of the differences

observed concerning some of the components identified by staining with different lectins.

Besides, the distribution of polysaccharides over EPS is not homogenous, leading to different results and cells which have a positive and negative interactions on the same sample (Dazzo & Brill, 1979).

The presence of different polysaccharides might be mediating the adhesion of *S. thermosulfidooxidans* to surfaces with a positive charge. It is known that the hydroxyl groups present in the polysaccharides of the EPS are able to interact with positive Ions in surfaces allowing the cell to bind to these surfaces (Weis, 1996). This property could also be used by the microorganism to attract positively charged iron ions to acquiring energy from them.

7.4. *S. thermosulfidooxidans* EPS characterization.

EPS was extracted from sulfur and pyrite grown cells. Depending on the energy source differences were observed in EPS composition (Teschke, 2005).

From both EPS extracted from sulfur and pyrite grown cells, it was observed that the humic acids were present in all the fractions in a high concentration (up to 20.7 % from the total) therefore, they could be considered as a key molecule playing an important role in the biofilm of *S. thermosulfidooxidans*. However, the concentration of humic acids may be due also to the decaying of organic matter from dead cells in the biofilm (Kreft & Wimpenny, 2001) and the degradation of yeast extract present in the medium. It is known that some of the components of the EPS may be produced normally as part of the lifecycle of the microorganism and their role in attachment is not clear (Whitfield, 2006).

The presence of polysaccharides in the EPS extracted from pyrite grown cells, also suggests they have a potential role in cell attachment to pyrite. It can be speculated that they are tightly bound to the cell surface, since it was not possible to detect them in the other fractions in levels as high as in the capsular second extraction. The

concentration of polysaccharides in the medium cultured with sulfur was not as high as expected, according to what was observed in the lectin staining, in which more polysaccharides were detected compared to the pyrite grown cells. Polysaccharides of EPS extracted from sulfur grown cells were detected only in colloidal and washed fractions (0.5 and 0.2 % from the total), although in some other cases it has been demonstrated that the higher percentage of organic carbon in the EPS does not correspond to polysaccharides. Moreover, the extraction reagent used may influence the polysaccharide solubility more than in the case of proteins, altering consequently the colorimetric measurements (Metzger et al., 2009).

Regarding the protein concentration in the EPS this may be affected by the protein source in the medium; it can also be influenced by the age of the biofilm, since the EPS matrix is a dynamic medium subjected to constant changes. For example cells are producing proteases in order to degrade some of the components and replace them for new and more stable molecules (Jiao et al., 2010; Flemming & Wingender, 2010); besides some researchers have found these differences are statistically significant through the biofilm age in some of the components like uronic acid (Mojica et al., 2007).

The differences between the measurements of the EPS components may also be due to the maturity of the biofilm (Jiao et al., 2010), depending on the age of biofilms the proportion of the components may change through time. The time for EPS extraction was chosen according to the initial attachment of cells over the surface, but this probably does not mean that the maturity of the biofilm is the same in both of them. Moreover, the energy substrate provides different amounts of energy which is later on used for synthesis of EPS and binary fission; this will finally lead to more cells producing more EPS. The measurements can also be altered by the presence of metal ions in the EPS, that is the main reason why the EPS is dialyzed to avoid the presence of those metals even if they are difficult to remove (Sand et al., 1995). This metal ions may change their concentration in the EPS depending on the substrate used to

grow the microorganism and also depends on the solubility of the metal in the medium (Jiao et al., 2010).

The differences observed between the fractions of EPS can be due to the threshold of EPS production which defines tightly bound EPS and loosely bound EPS; all these make differences in terms of amount and composition (Li & Yang, 2007). Despite of these reasons, probably the main one to explain the differences between the EPS extracted from pyrite and sulfur grown cells is the one related with the energy source and the support (Teschke, 2005; Gehrke et al., 1998a).

The percentages of cell lysis for the cells grown on pyrite or sulfur during EPS extraction were much less than 1 % of the initial culture. This means that the measurements in the samples are corresponding to the composition of the EPS itself and no other cell compounds from the inside are interfering significantly to the signal of the spectrophotometric assays. These results are in agreement with the fact that DNA measurements in the extracted EPS samples were under the detection limits.

In the case of the medium with ferrous iron, the absence of EPS in the cells cultured in this medium can be due to the absence of an interacting surface for supporting its growth. It is known that EPS is a key for the adhesion and bioleaching of ores and therefore if there is no support where the cells can get attached to, the production of EPS would be low or even there will not be production of EPS at all (Gehrke et al., 1998a). Similar results were obtained for EPS extracted from planktonic cells cultured in sulfur, where the dry weight was approximately 10 times lower and in the case of some fractions no dry weight was detected at all.

The results of EPS analysis are showing different rates of EPS production under different cellular states. Moreover the characteristics and composition of this EPS is different depending on the surface and the cellular state, sessile or planktonic. The EPS have been shown to be key molecules for adhesion to surfaces and probably if *S. thermosulfidooxidans* was not able to produce any EPS it would not be able to attach to any surface (Gehrke et al., 1998b; Sampson et al., 2000; Arredondo et al., 1994;

Watnick & Kolter, 1999). Among the composition of the EPS, humic acids, polysaccharides and proteins were found which are probably conferring and stablishing Lewis acids forces, ionic forces and van der Waal´s forces that are necessary for the attachment of *S. thermosulfidooxidans* to metal sulfide surfaces (Gehrke et al., 1998b; Becker et al., 2011); and without the EPS a further adhesion of *S. thermossulfidooxidans* to a surface, would be impaired, thus bioleaching rates would be lower (Ding et al., 2007; Dazzo & Brill, 1979; Arredondo et al., 1994)

However, there is still a high percentage of unknown compounds in EPS extracted from cultures grown in pyrite or sulfur. The analysis of the full spectra suggests the presence of aliphatic compounds (Harris, 1999) and concerning EPS composition it is known that colonic acids, teichoic acids, uronic acids, lipids, metal ions, polysialic acids and residues of phosphate can also be found (Sand et al., 1995; Whitfield, 2006).

7.5. Bioinformatics.

The bioinformatics search on the DOE Joint Genome Institute showed many strong candidate genes which are probably related to EPS biosynthesis. However, only three of them were chosen because of their genetic context in the genome. According to the context, glycosiltranferases and ABC transporters involved in the exportation of polysaccharides from the EPS were present, making them the strongest candidates for being involved in the processes mentioned before (Cuthbertson et al., 2010; Valenzuela et al., 2006).

The sequence of Sulth_1632 was taken for running alignments with the whole genome of *Sulfobacillus.* The results showed that there are at least 25 sequences over *S. thermosulfidooxidans* genome with some level of similarity. Since these sequences are not identical and some of them have a low level of similarity (appendix 9.7), choosing a sequence for further analysis is not an easy task. In comparison, the other sequences 1631 and 1635 showed 1 and 4 matched sequences (including the query) aligned with the whole genome.

DNA and RNA extraction was performed but no successful amplification was achieved. The primers designed in this thesis were made from carefully chosen genes; therefore it is thought that the concentration of DNA and RNA in the samples was not enough for a successful amplification. Then the primers designed in this study could be suggested for further studies but it is recommended to reconsider the extraction method of DNA and RNA in addition, it is recommendable to obtain a higher cell concentration in the culture.

8. Conclusions and future perspectives.

S. thermosulfidooxidans DSM 9293 is able to grow on medium with different energy sources such as sulfur and iron. Nevertheless, it presents a better growth on iron sulfate than in reduced forms of sulfur such as tetrathionate and thiosulfate. Higher growth can be achieved under mixotrophic conditions than in heterotrophic conditions. The ferrous iron might play a key role in the growth of *S. thermosulfidooxidans* since the medium lacking of ferrous iron showed the lowest cell concentration. The mechanisms underlying this role of ferrous iron should be studied in detail.

Acording to the results achieved in this thesis, depending on the energy source, the EPS produced by *S. thermosulfidooxidans* changes its composition including the lectins present in each one; the amount of EPS produced also changes depending on the energy source. Although *S.thermosulfidooxidans* is able to grow in medium with ferrous iron, the EPS production under these conditions is low. When cultured in iron sulfate and sulfur, *S. thermosulfidooxidans* produces lower EPS under planktonic state compared to sessile state of cells grown in pyrite and sulfur.

Among the detectable compounds of the EPS, humic acids are in the highest concentration. Nevertheless, the unknown substances are in the major percentage. Polysaccharides might be present tightly bound to the membrane and its presence depends on the energy source. Although the polysaccharide production in sulfur grown cells is more diverse compared to the pyrite grown cells, as observed by the lectin stainings, the amount of polysaccharides produced is higher for cells grown in pyrite. In order to identify some of the unknown compounds present in the EPS more specialized techniques like HPLC and mass spectrometry, should be employed for this analysis.

According to literature and sequence alignments, proteins belonging to the ABC transporters are similar. Besides, these sequences are spread all over *S. thermosulfidooxidans* genome. More specific probes, such as Taqman, should be

employed in order to obtain more accurate results. It is also strongly recommended to align the sequences to take the mispairng zones in order to design primers.

References

1991. Validation of the publication of new names and new combinations previously effectively published outside the IJSB, International journal of systematic bacteriology 41: 178-179.

Aguilera, Souza-Egipsy, Martin-Úriz & Amils, 2008. Extraction of extracellular poilymeric substances from extreme acidic microbial biofilms, Applied Microbiology and Biotechnology 78: 1079-1088.

Aljanabi& Martinez, 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques, Nucleic Acids Research 25: 4692-4693.

Altschul, Gish, Miller, Myers & Lipman, 1990. Basic local alignment search tool, Journal of molecular biology 215: 403-410.

Arredondo, García & Jerez, 1994. Partial removal of lipopolysaccharide from *Thiobacillus ferroxidans* affects its adhesion to solids, Applied and Environmental Microbiology 60: 2846-2851.

Barreto, Gehrke, Harneit, Sand, Jedlicki & Holemes, 2005. Unexpected insights into biofilm formation by *Acidithiobacillus ferrooxidans* revealed by genome analysis and experimental approaches. 817-825.

Becker, Gorham, Shiers & Watling, 2011. *In situ* imaging of *Sulfobacillus thermosulfidooxidans* on pyrite under conditions of variable pH using tapping mode atomic force microscopy., Process Biochemistry 46: 966-976.

Bellenberg, Leon-Morales, Sand & Vera, 2012. Visualization of capsular polysaccharide induction in *Acidithiobacillus ferrooxidans*, Hydrometallurgy 129: 82-89.

Bevilaqua, Leite, Garcia & Tuovinen, 2002. Oxidation of chalcopyrite by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* in shake flask., Process Biochemistry 38: 587-592.

Blake II, Shute & Howard, 1994. Solubilization of minerals by bacteria: electrophoretic mobility of *Thiobacillus ferroxidans* in the presence of iron, pyrite and sulfur, Applied and Environmental Microbiology 60: 3349-3357.

Bogdanova, Tsaplina, Kondrat'eva, Duda, Suzina, Melamud, Tourova & Karavaico, 2006. *Sulfobacillus thermotolerans* sp. nov., a thermotolerant, chemolithotrophic bacterium., International Journal of Systematic and Evolutionary Microbiology 56: 1039-1042.

Bomchil, Watnick & Kolter, 2003. Identification and characterisation of a *Vibrio cholerae* gene, *mbaA*, involved in the maintenance of biofil architechture, Journal of bacteriology 185: 1384-1390.

Bond, Smriga & Banfield, 2000. Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site., Applied and Environmental Microbiology 66: 3842-3849.

Branda, Chu, Kearns, Losick & Kolter, 2006. A major protein component of the *Bacillus subtillis* biofilm matrix, Molecular microbiology 59: 1229-1238.

Bridge& Johnson, 2000. Reductive dissolution of ferric iron minerals by *Acidiphilium* SJH, Geomicrobiology Journal 17: 193-206.

Brockmann, Arnold, Schweder & Bernhard, 2010. Visualizing acidophilic microorganisms in biofilm communities using acid stable fluorescence dyes, Journal of Fluorescence 20: 943-951.

Buleav, Pivovarova, Melamud, Tsaplina, Zhuravleva & Kondrat'eva, 2011. Polymorphism of *Sulfobacillus thermosulfidooxidans* strains dominating in processes of high-temperature oxidation of gold-arsenic ore concentrate, Microbiology 80: 326-334.

Burton, 1955. A study of the conditions and mechnisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid, Biochemical Journal 62: 315-323.

Caldwell, MacLean & Norris, 2007. Ribulose bisphosphate carboxylase activity and Calvin cycle gene cluster in *Sulfobacillus* species., Microbiology 153: 2231-2240.

Chae& Schraft, 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains, International journal of food microbiology 62: 103-111.

Chi, Valenzuela, Beard, Mackey, Shabanowitz, Hunt & Jerez, 2007. Periplasmic proteins of the extremophile *Acidithiobacillus ferrooxidans*, The American Society for Biochemistry and Molecular Biology 6: 2239-2251.

Clark& Norris, 1996. *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed-culture ferrous iron oxidation with *Sulfobacillus* species., Microbiology 142: 785-790.

Cuthbertson, Kos & Whitfield, 2010. ABC transporters involved in export of cell surface glycoconjugates, Microbiology and Molecular Biology Reviews 74: 341-362.

Dazzo& Brill, 1979. Bacterial polysaccharide which binds *Rhizobium trifolii* to clover root hairs, Journal of bacteriology 137: 1362-1373.

Ding, Gao, Wu, Zhang, Wang & Qiu, 2007. Jarosite-type precipitates mediated by YN22, *Sulfobacillus thermosulfidooxidans*, and their influences on strain., Transactions of nonferrous metals society of china 17: 1038-1044.

Donlan, 2002. Biofilms: microbial life on surfaces, Emerging infectious diseases 8: 881-890.

Dubois, Gilles, Hamilton, Rebers & Smith, 1956. Colorimetric method for determination of sugars and related substances, Analytical Chemistry 28: 350-356.

Egorova, Tsaplina, Zakharchuk, Bogdanova & Krasil'nikova, 2004. Effect of cultivation conditions on the growth and activities of sulfur metabolism enzymes and carboxylases of *Sulfobacillus thermosulfidooxidans* subsp. *asporogenes* strain 41., Applied biochemistry and microbiology 40: 381-387.

Flemming& Wingender, 2010. The biofilm matrix, Nature Reviews Microbiology 8: 623-633.

Frolund, Palmgren, keiding & Nielsen, 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin, Water Research 30: 1749-1758.

Gehrke, Telegdi, Thierry & Sand, 1998a. Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching, Applied and Environmental Microbiology 64: 2743-2747.

Gehrke, Telegdi, Thierry & Sand, 1998b. Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching., Applied Environmental Microbiology 64: 2743-2747.

Ghauri, Okibe & Johnson, 2007. Attachment of acidophilic bacteria to solid surfaces: The significance of species and strain variation, Hydrometallurgy 85: 72-80.

Harris, 1999. Quantitative chemical analysis. Reverté.

Jiao, Cody, Harding, Wilmes, Schrenk, Wheeler, Banfield & Thelen, 2010. Characterization of extracellular polymeric substances from acidophilic microbial biofilms, Applied and Environmental Microbiology 76: 2916-2922.

Karatan& Watnick, 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms, Microbiology and Molecular Biology Reviews 73: 310-347.

Karavaiko, Bulygina, Tsaplina, Bogdanova & Chumakov, 1990. *Sulfobacillus thermosulfidooxidans*: a new linage of bacterial evolution?, FEBS letters 261: 8-10.

Karavaiko, Krasil'nikova, Tsaplina, Bogdanova & Zakharchuk, 2001. Growth and carbohydrate metabolism of sulfobacilli, Microbiology 70: 245-250.

Karavaiko, Zakharchuk, Bogdanova, Egorova, Tsaplina & Krasil'nikova, 2002. The enzymes of carbon metabolism in the thermotolerant bacillar strain K1, Microbiology 71: 651-656.

Kock& Schippers, 2008. Quantitative microbial community analysis of three different sulfidic mine tailing dumps generating acid mine drainage., Applied and Environmental Microbiology 74: 5211-5219.

Krasil'nikova, Zakharchuk, Egorova, Bogdanova, Zhuravleva & Tsaplina, 2010. Regulation of metabolic pathways in Sulfobacilli under different aeration regimes, Microbiology 79: 147-152.

Kreft& Wimpenny, 2001. Effect of EPS on biofilm structure and function as revealed by an individual-based model of biofilm growth, Water Science and Technology 43: 135-142.

Li& Yang, 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge, Water Research 41: 1022-1030.

Lowry, Rosenbrough, Farr & Randall, 1951. Protein measurement with the folin phenol reagent, Journal of Biological Chemistry 193: 265-275.

Mackintosh, 1978. Nitrogen fixation by *Thiobacillus ferroxidans*., Journal of General Microbiology 105: 215-218.

Matsukawa& Greenberg, 2004. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development, Journal of bacteriology 186: 4449-4456.

Mendez, Neilson & Maier, 2008. Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site., Applied and Environmental Microbiology 74: 3899-3907.

Metzger, Lankes, Fischpera & Frimmel, 2009. The concentration of polysaccharides and proteins in EPS of *Pseudomonas putida* and *Aureobasidum pulludans* as revealed by ^{13}C CPMAS NMR spectroscopy, Applied Microbiology and Biotechnology 85: 197-206.

Mojica, Elsey & Cooney, 2007. Quantitative analysis of biofilms EPS uronic acid content, Journal of Microbiological Methods 71: 61-65.

Morikawa& Yanagida, 1981. Visualization of individual DNA molecules in solution by light microscopy: DAPI staining method, Journal of biochemistry 89: 693-696.

Muravyov, Pivovarova, Tourova, Buleav, Fomchenko & Kondrat´eva, 2010. Identification of the dominant bacterium of two-stage biooxidation of gold-arsenic concentrate, Microbiology 79: 342-348.

NG& Dawes, 1973. Chemostat studies on the regulation of glucose metabolism in *Pseudomonas aeruginosa* by citrate, Biochemica Journal 132: 129-140.

Nicomrat, Dick & Tuovinen, 2006. Microbial populations identified by fluorescence in situ hybridization in a constructed wetland treating acid coal mine drainage., Journal of Environmental Quality 35: 1329-1337.

O´Toole, Kaplan & Kolter, 2000. Biofilm formation as microbial development, Annual Review of Microbiology 54: 49-79.

Rawlings, 2002. Heavy metal mining using microbes, Annual Review of Microbiology 56: 65-91.

Rozen& Skaletsky, 2000. Primer3 on the www for general users and for biologist programmers. 365-386.

Sampson, Phillips & Blake, 2000. Influence of the attachment of acidophilic bacteria during the oxidation of mineral sulfides., Minerals engineering 13: 373-389.

Sand& Gehrke, 2006. Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron (III) ions and acidophilic bacteria., Research in Microbiology 157: 49-56.

Sand, Gehrke, Hallmann & Schippers, 1995. Sulfur chemistry, biofilm, and the (in)direct attack mechanism - a critical evaluation of bacterial leaching, Applied Microbiology and Biotechnology 43: 961-966.

Schippers& Sand, 1999. Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur, Applied and Environmental Microbiology 65: 319-321.

Schrenk, Edwards, Goodman, Hamers & Banfield, 1998. Distribution of *Thiobacillus ferroxidans* and *Leptospirillum ferroxidans*: implications for the generation of acid mine drainage, Science 279: 1519-1522.

Skoog& West, 1963. Fundamentals of analytica chemistry. New york.

Slifkin& Doyle, 1990. Lectins and Their Application to Clinical Microbiology, Clinical Microbiology Reviews 3: 197-218.

Subramanian, Yan, Tyagi & Surampalli, 2011. Characterization of extracellular polymeric substances (EPS) extracted from both sludge and pure bacterial strains isolated from wastewater sludge for sludge dewatering.

Teschke, 2005. Volume of extracellular polymeric substances coverage of individual *Acidithiobacillus ferrooxidans* bacterium measured by atomic force microscopy, Microscopy Research and Technique 67: 312-316.

Tourova, Poltoraus, Lebedeva, Tsaplina, Bogdanova & Karavaico, 1994. 16S Ribosomal RNA (rDNA) sequence analysis and phylogenetic position of *Sulfobacillus thermosulfidooxidans*, Systematic and applied microbiology 17: 509-512.

Valenzuela, Chi, Beard, Orell & Guiliani, 2006. Genomics, metagenomics and proteomics in biomining microorganisms, Biotechnology Advances 24: 197-211.

Vera, Bellenberg, Sand, Denis & Bonnefoy, 2009. Characterization of biofilm formation by the bioleaching acidophilic bacterium *Acidithiobacillus ferrooxidans* by microarray transcriptome analysis., Advanced Materials Research 71-73: 175-178.

Watnick& Kolter, 1999. Steps in the development of *Vibrio cholerae* El Tor biofilm, Molecular microbiology 34: 586-595.

Weis, 1996. Structural basis of lectin-carbohydrate recognition, Annual review of biochemistry 65: 441-473.

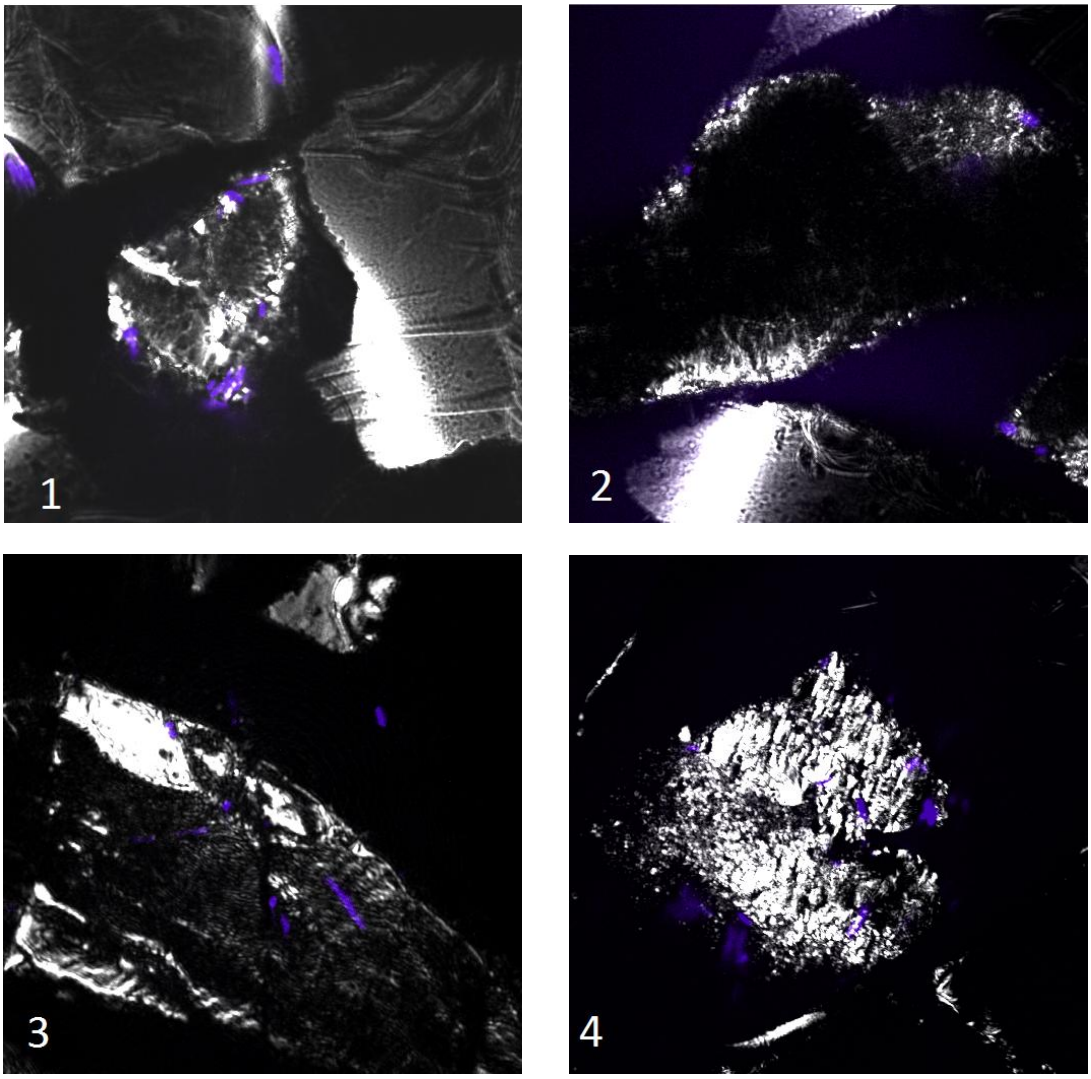
Whitfield, 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*, The anual review of biochemistry 75: 39-68.

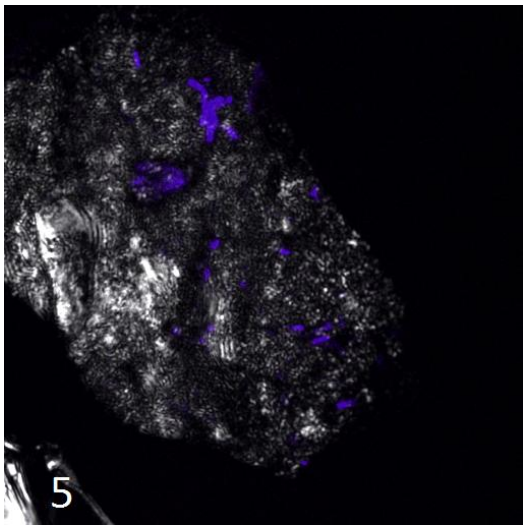
Wuertz, Spaeth, Hinderberger, Griebe, Flemming & Wilderer, 2001. A new method for extraction of extracellular polymeric substances from biofilms and actived sludge suitable for direct quantification of sorbed metals., Water Science and Technology 43: 25-31.

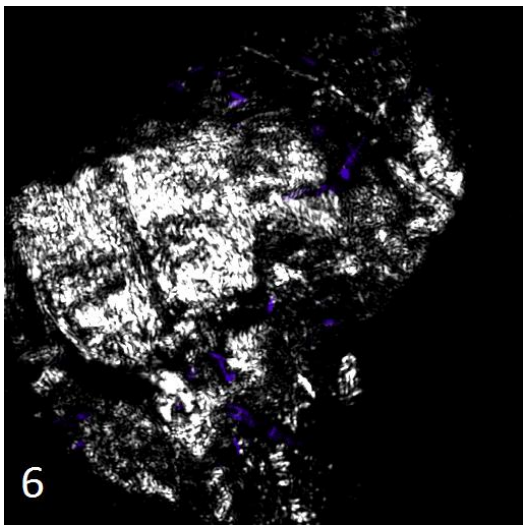
Yildiz& Schoolnik, 1999. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation, Proceedings of the national academy of science 96: 4028-4033.

10. Appendix

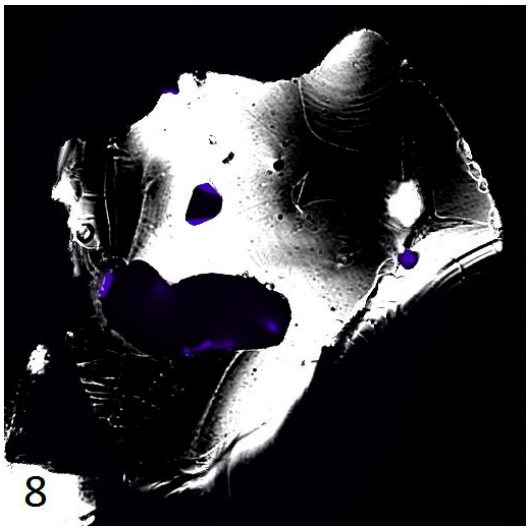
10.1. Attachment of *S. thermosulfidooxidans* to pyrite. (the number in the corner correspond to the day when sample was taken from culture to stain).

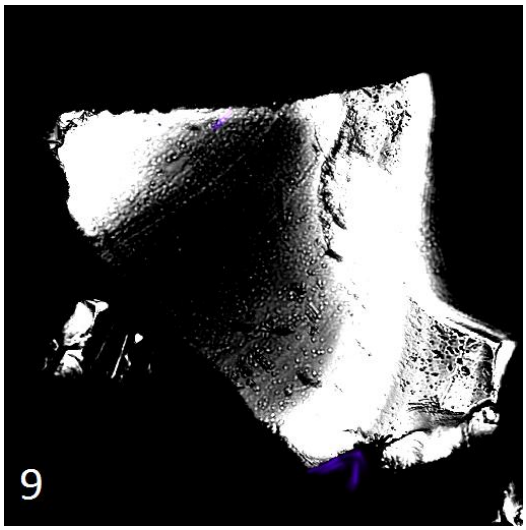


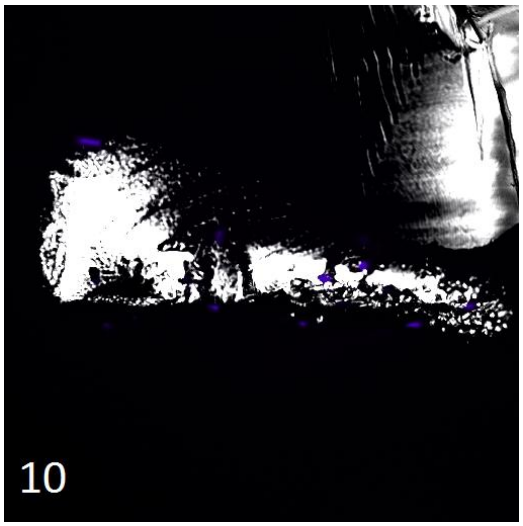

5

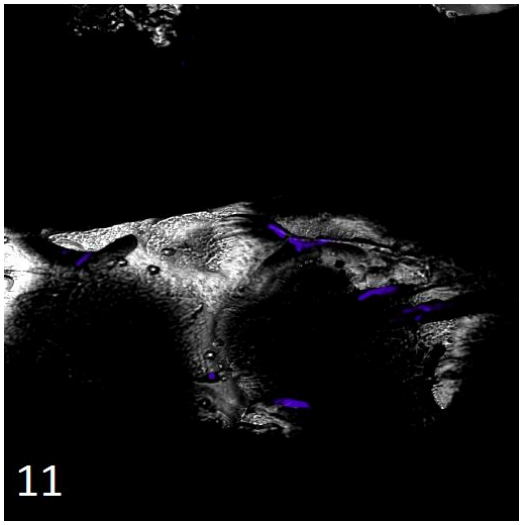

6


7

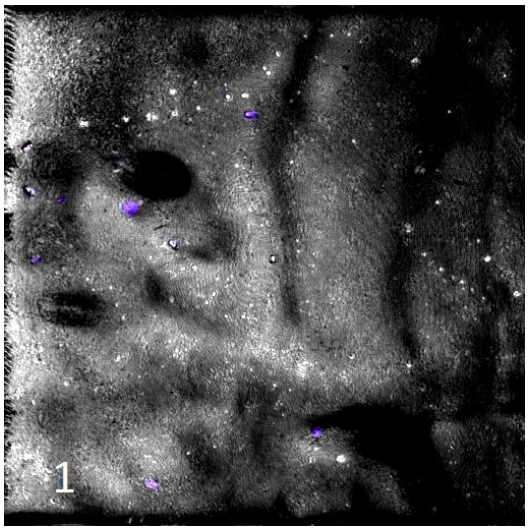

8

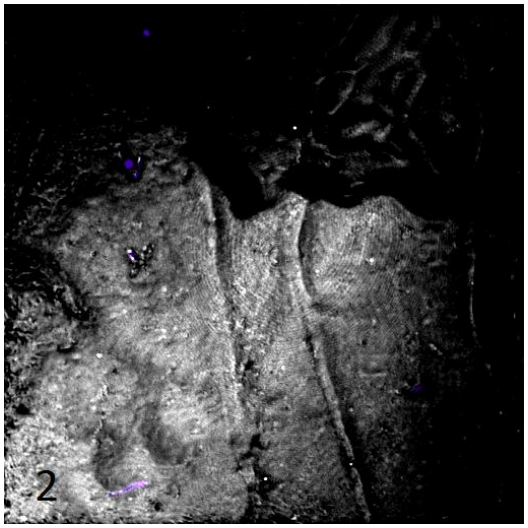

9

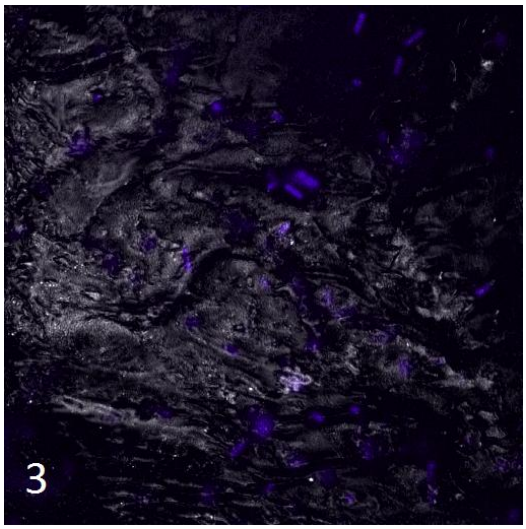

10

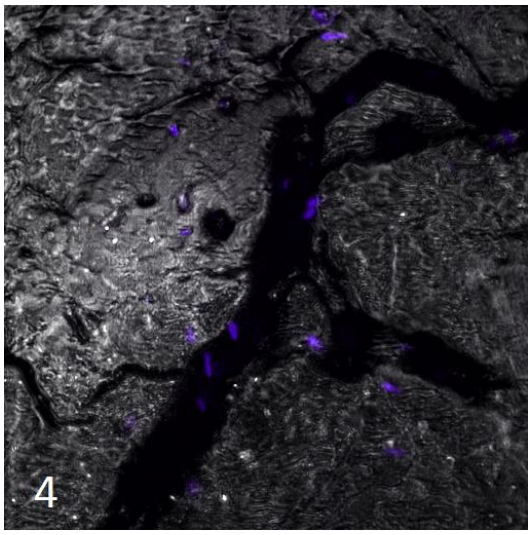


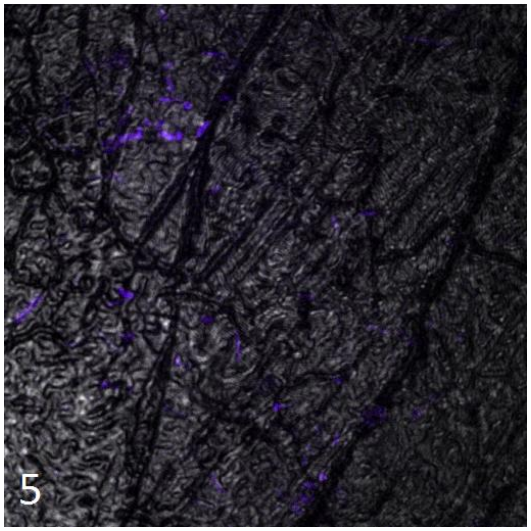
10.2. Attachment of *S.thermosuilfidooxidans* to sulfur. (the number in the corner correspond to the day when sample was taken from culture to stain).

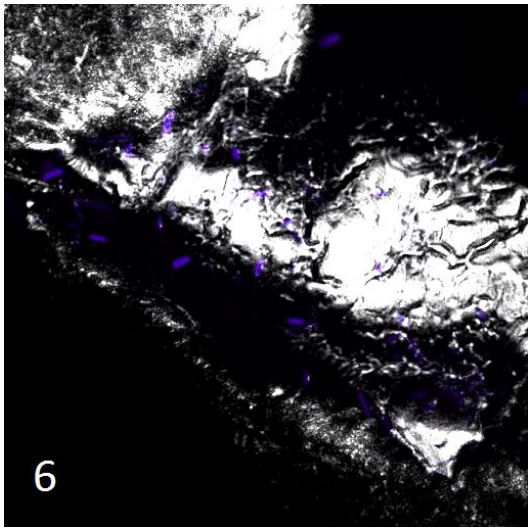


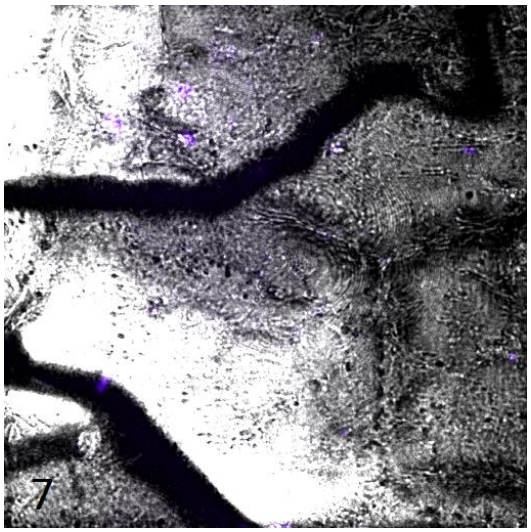


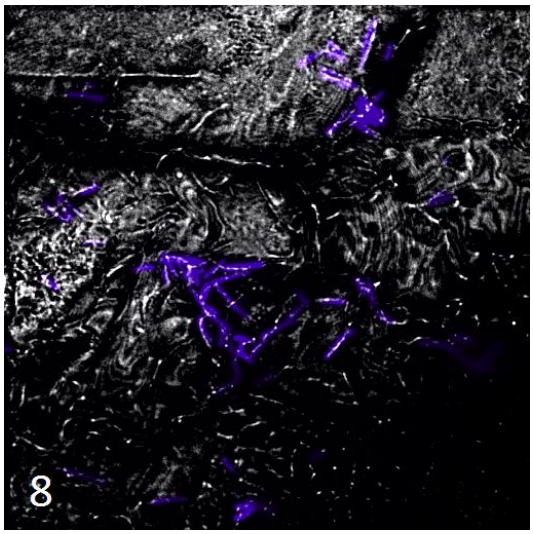

3

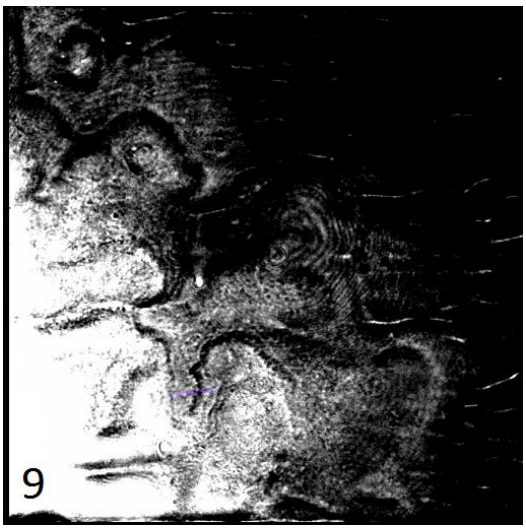

4

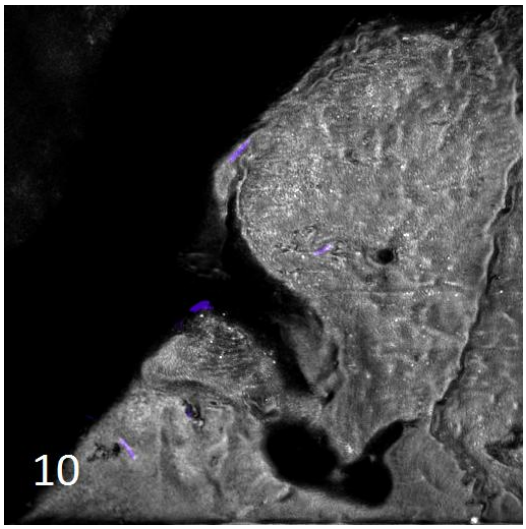

5

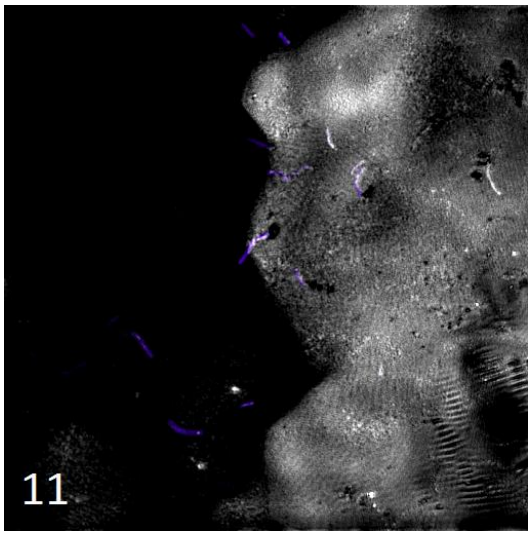

6


7


8

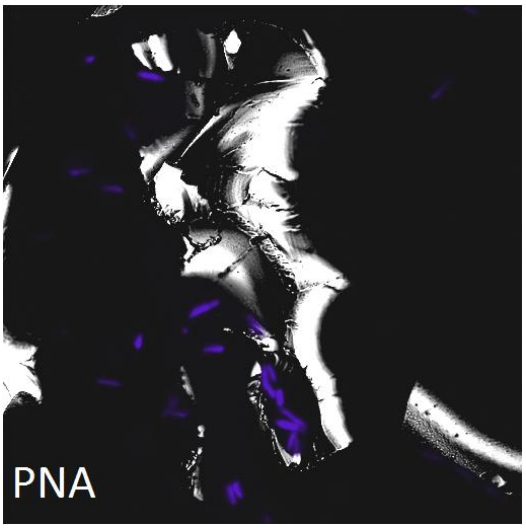


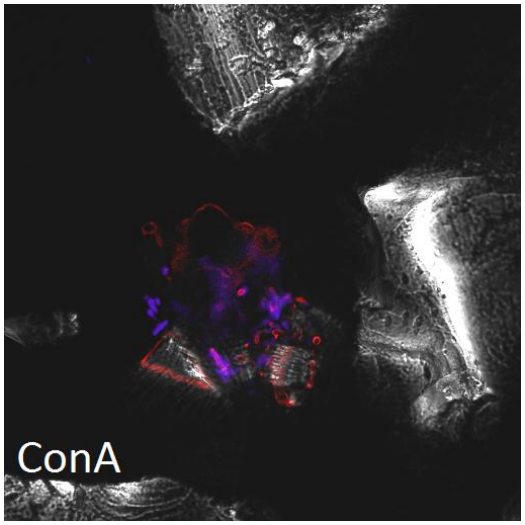


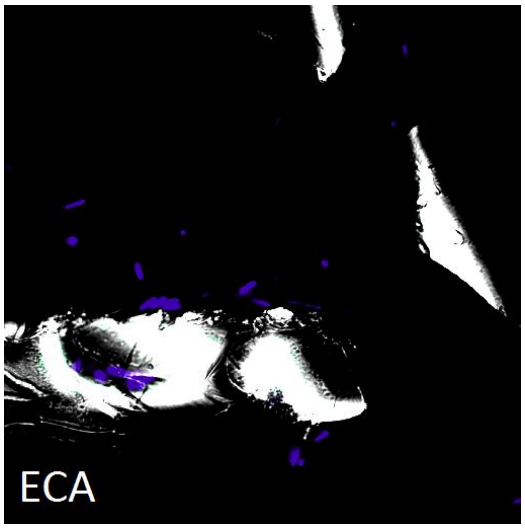


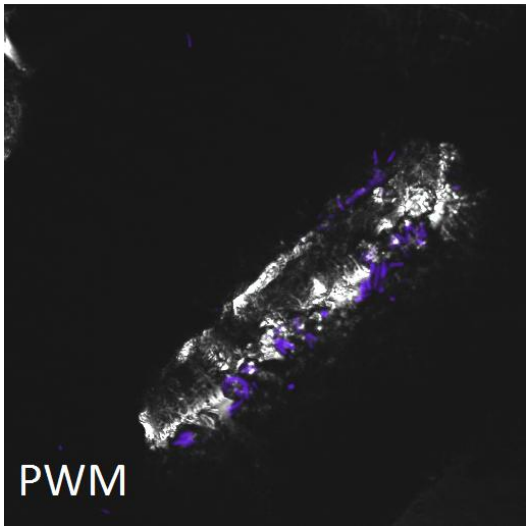
10.3. Lectin stainings of pyrite grown cells.

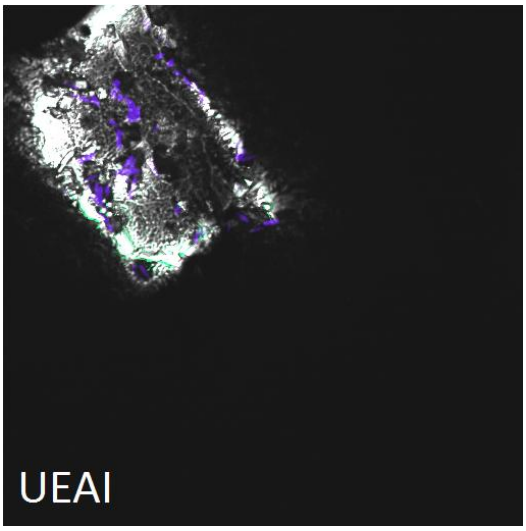
10.3.1. Lectin stainings of pyrite grown cells (sessile)

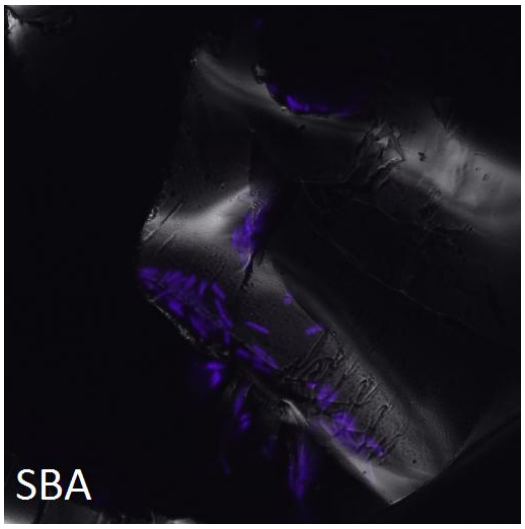

PNA

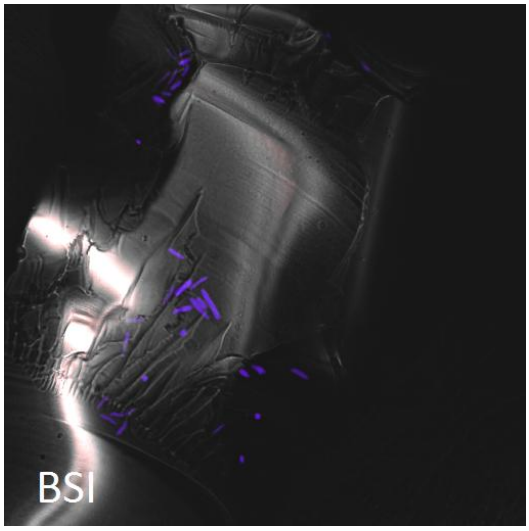

ConA


ECA

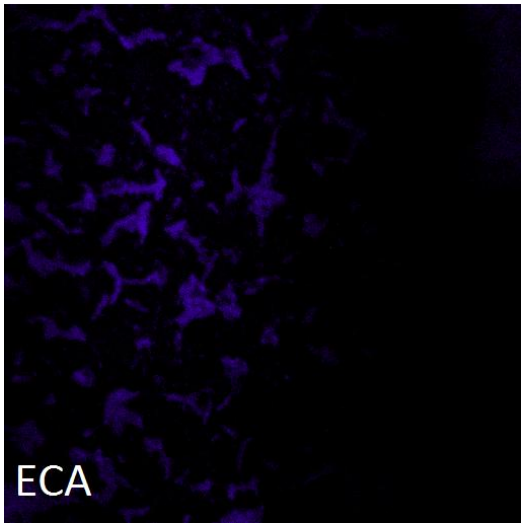

PWM

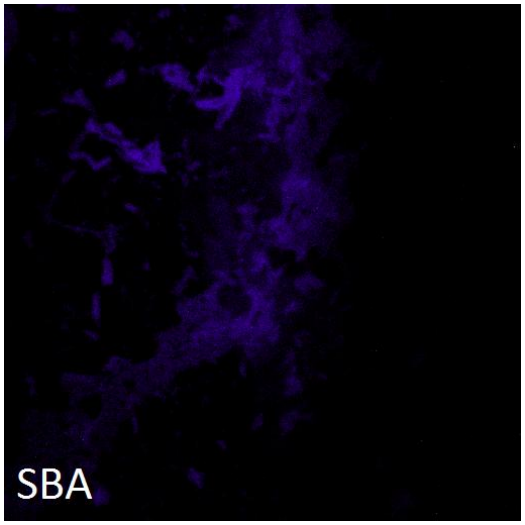

UEAI

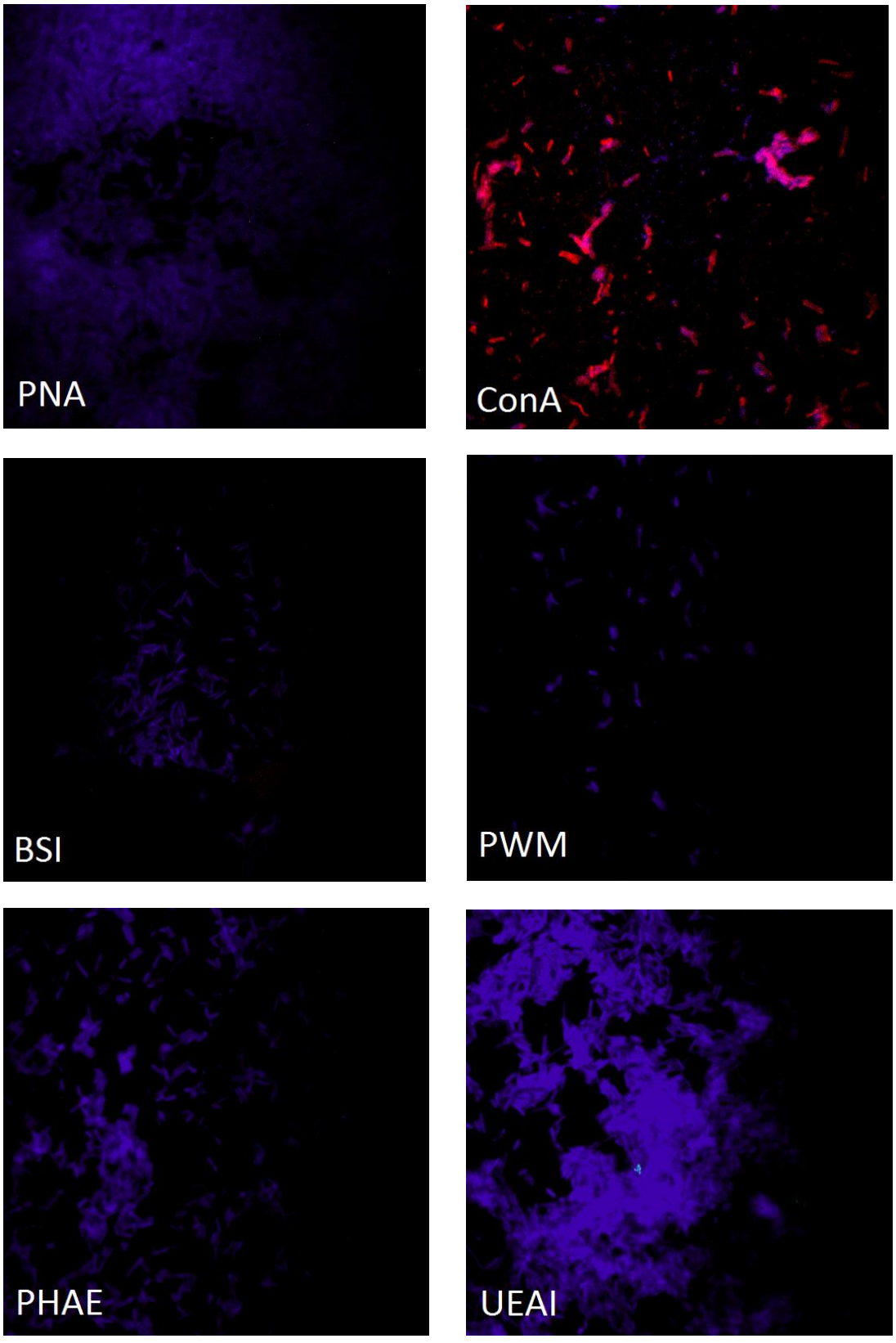

SBA

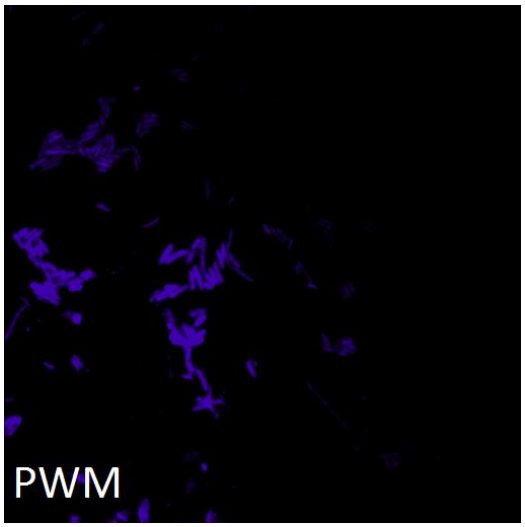


10.3.2. Lectin stainings of pyrite grown cells (planctonic)



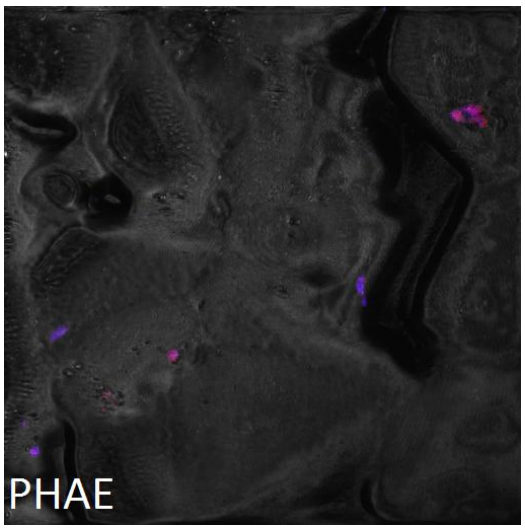


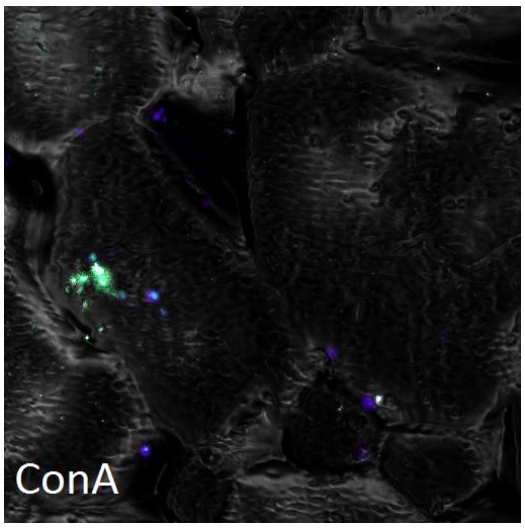

PNA
ConA
BSI
PWM
PHAE
UEAI

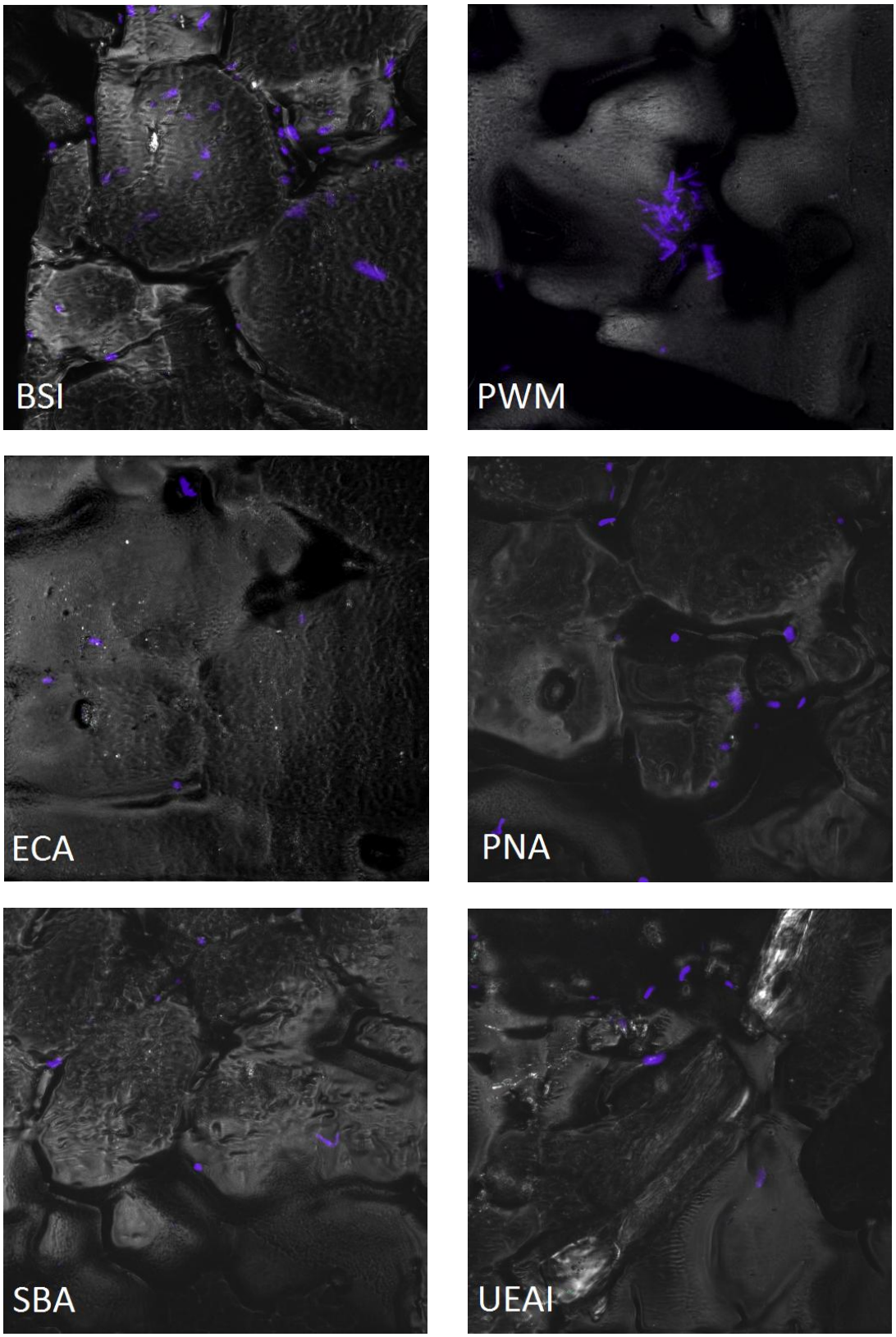


10.4. Lectin stainings of sulfur grown cells.

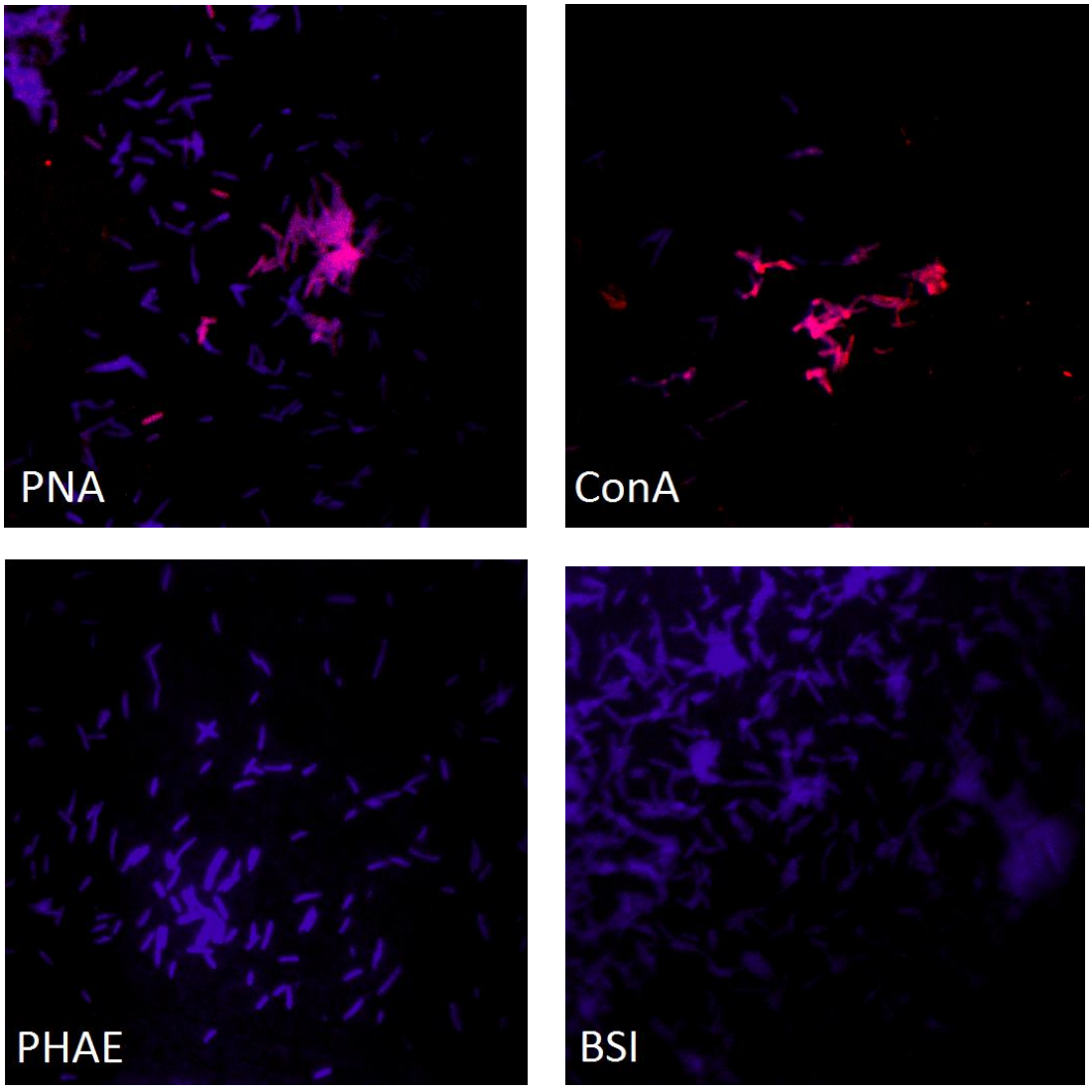
10.4.1. Lectin stainings of sulfur grown cells (sessile)

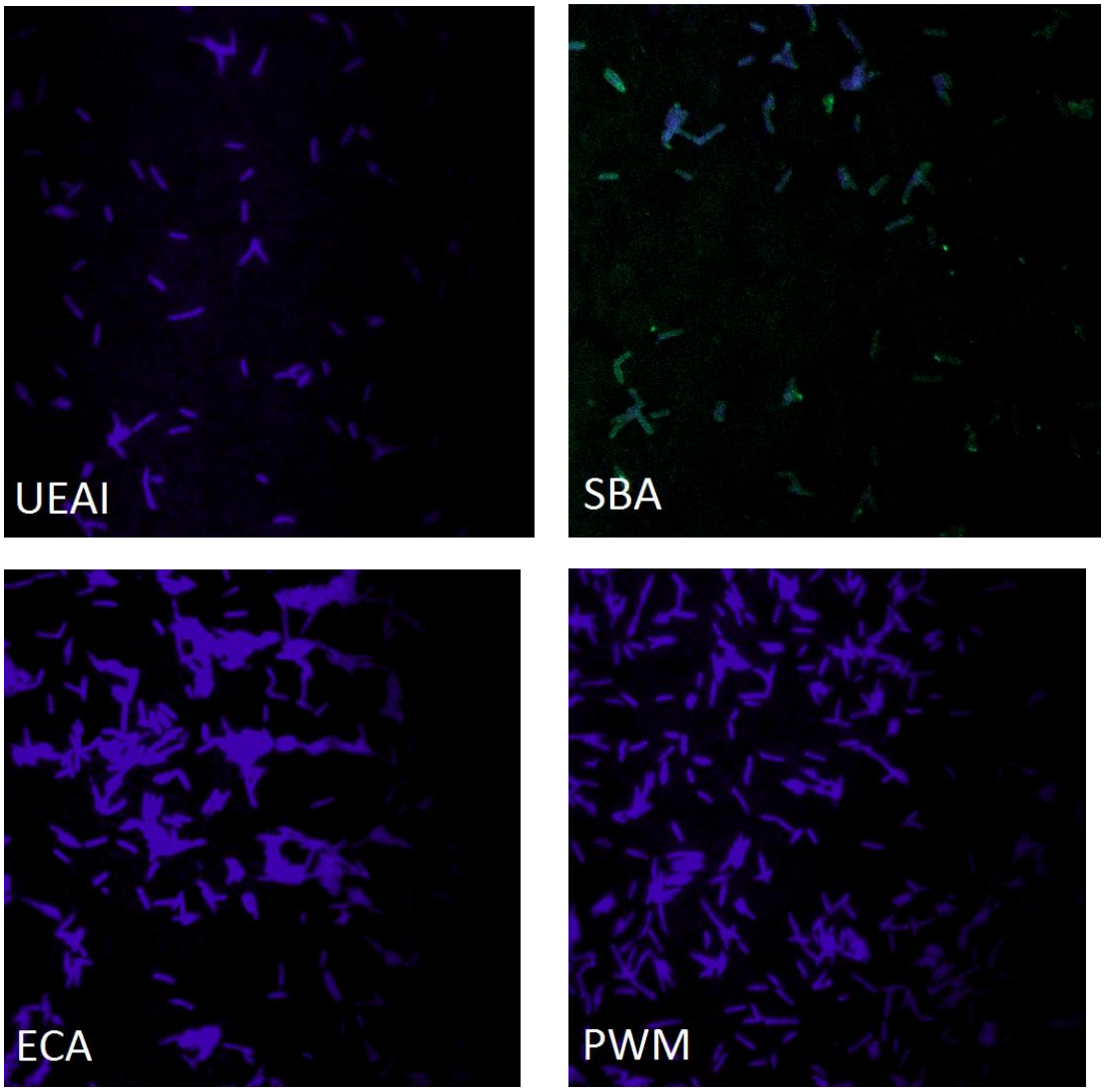





BSI
PWM
ECA
PNA
SBA
UEAI

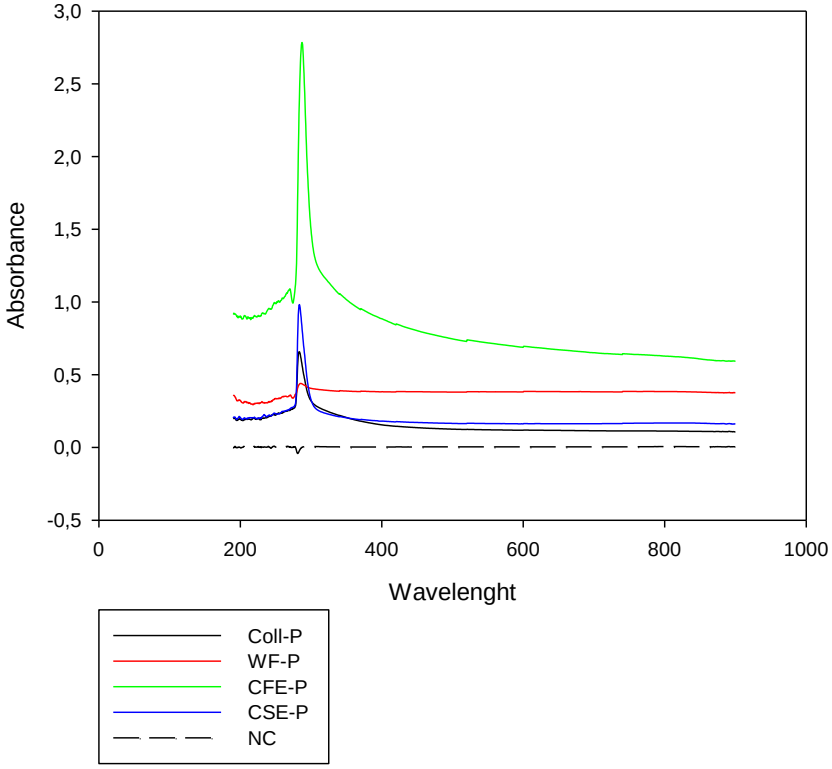
10.4.2. Lectin staining of sulfur grown cells (planctonic)



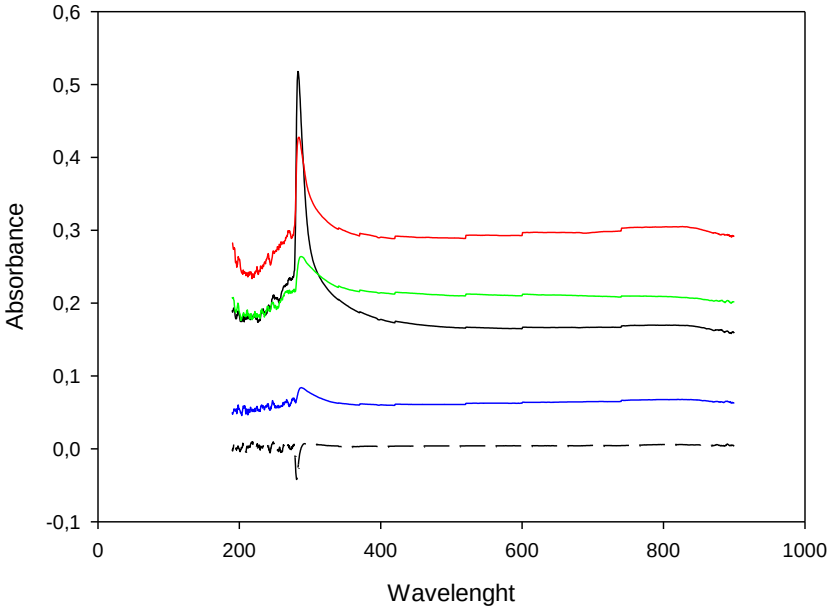


10.5. Whole spectra of EPS samples.

EPS extracted from pyrite grown cells



EPS extracted from sulfur grown cells



10.6. BLAST of reference sequences against *S. thermosulfidooxidans* genome.

Literature	Query	Number of hits	Lowest E-value	Description E-value	Greatest identity %	Greatest positive %	Greatest hit length
Whitfield 2006	ABC transporter ATPase	37	8,23E-20	contig1	55	85	729
	ABC transporter	48	3,82E-35	contig1	63	94	909
	CpxA [Actinobacillus pleuropneumoniae]	36	2,65E-30	contig1	50	75	732
	KpsT [Escherichia coli]	44	1,38E-32	contig1	55	81	705
	malK [Escherichia coli]	48	4,99E-82	contig1	52	72	1107
	MsbA [Escherichia coli]	38	2,61E-65	contig1	55	75	1794
	polysaccharide export protein [Sinorhizobium fredii NGR234]	32	4,29E-29	contig1	65	76	681
	rfbI [Vibrio cholerae]	46	9,18E-43	contig1	72	89	720
	TagH [Pseudomonas fluorescens F113]	43	4,67E-61	contig1	62	68	768
	Wzt [Escherichia coli]	21	1,83E-51	contig1	46	60	678
Bomchil 2003	VC0701	29	0,53	contig1	80	83	153
	VC0702	32	0,1	contig1	89	100	159
	VC0703	74	1,78E-42	contig1	67	83	546
	VC0704	15	0,02	contig1	67	87	162
Donlan 2002	ALG44	33	0,48	contig1	71	86	189
	ALGC	75	2,34E-102	contig1	82	100	588
	ALGD	22	0,72	contig1	100	100	159
	EPSA-EPSM	76	2,64E-40	contig1	82	91	363
Branda 2006	EPSA-EPSM	69	2,64E-40	contig1	82	87	363
	TASA	12	1,8	contig1	82	91	105
Matsukawa 2004	PA1381	42	0,05	contig1	71	86	204
	PA1384	86	6,94E-31	contig1	91	91	291

	PA1388	75	8,20E-03	contig1	82	91	165
	PA1390	121	1,56E-15	contig1	71	83	297
	PA3552	43	2,00E-82	contig1	71	74	507
	PA3553	33	6,59E-09	contig1	73	87	165
	PA3558	3	4,33	contig2	56	63	141
Yildiz 1999	VPS18	20	1,52	contig1	64	91	105
	VPS21	30	1,95E-19	contig1	86	100	189
	VPS3	21	2,93E-07	contig1	72	83	111
	VPS32	51	7,41E-09	contig1	69	80	321
	VPS38	20	0,72	contig1	67	79	132
	VPS39	22	0,12	contig1	75	100	108
	VPS4	16	0,7	contig1	78	78	123
	VPS50	32	0,08	contig1	86	100	129
	VPS54	55	2,87E-08	contig1	81	92	183
	VPS59	4	0,7	contig2	56	75	81
	VPS65	11	0,57	contig1	70	83	75
	VPS69	24	0,09	contig2	67	94	108
	VPS70	40	0,42	contig2	88	100	153
	VPS73	27	0,31	contig1	82	100	129
	VPS74	14	1,74	contig1	70	93	141
	VPS9	14	0,53	contig1	75	83	75

Sugar ABC transporter ATPase [Nitrosomonas europaea ATCC 19718]

Hit	Description	E-value	Score	Hit start	Hit end	%Gaps
0	contig1	8,23E-20	224	1673429	1674073	1
0	contig1	4,27E-11	149	2663665	2664273	9
0	contig1	5,62E-10	139	633228	633686	9
0	contig1	1,07E-09	137	1345060	1345629	9
0	contig1	3,62E-08	124	2870144	2870728	13
0	contig1	2,46E-07	117	2651649	2652107	6
0	contig1	6,02E-07	113	2171234	2171875	8
0	contig1	1,27E-05	102	1859546	1859728	3
0	contig1	1,53E-05	101	1440362	1440727	12
0	contig1	2,16E-05	100	1264982	1265491	15

0	contig1	5,23E-05	97	1933205	1933777	9
0	contig1	5,63E-05	96	1238901	1239026	0
0	contig1	6,12E-05	96	3023691	3024290	10
0	contig1	1,17E-04	93	2848359	2848985	13
0	contig1	1,25E-04	93	1706057	1706518	16
0	contig1	1,28E-04	93	1705027	1705743	13
0	contig1	3,71E-04	89	1919031	1919702	14
0	contig1	4,24E-04	89	2000464	2000928	10
0	contig1	4,93E-04	88	570210	570386	0
0	contig1	6,88E-04	87	2508599	2508721	7
0	contig1	8,48E-04	86	2331573	2332211	11
0	contig1	9,37E-04	86	2402851	2403459	12
0	contig1	1,13E-03	85	1030666	1030797	0
0	contig1	2,53E-03	82	571402	572130	14
0	contig1	2,75E-03	82	2006616	2007251	11
0	contig1	3,65E-03	81	2847497	2848135	6
0	contig1	0,01	76	761773	762246	8
0	contig1	0,04	72	1031659	1032030	1
0	contig1	0,04	71	3218638	3219129	13
0	contig1	0,05	71	1818871	1819359	15
0	contig1	0,05	71	2405105	2405230	0
0	contig1	0,05	71	1147051	1147179	0
0	contig1	0,07	70	1932902	1933030	0
0	contig1	0,07	70	700415	701086	11
0	contig1	0,08	69	2869290	2869712	8
0	contig1	0,09	69	2742317	2742430	0
0	contig1	0,13	67	2330917	2331183	2
0	contig1	0,19	66	2008916	2009011	0
0	contig1	0,22	65	227461	227646	0

0	contig1	0,37	63	2176090	2176191	0
0	contig1	0,4	63	3498056	3498115	0
0	contig1	0,81	60	1920061	1920198	4
0	contig1	1,27	59	652158	652256	0
0	contig1	1,41	58	761315	761482	0
0	contig1	1,77	57	3331010	3331135	0
0	contig1	1,86	57	2649777	2649971	23
0	contig1	2,29	56	3220557	3220670	0
0	contig1	2,62	56	2640276	2640362	0
0	contig1	3,17	55	403025	403225	0
0	contig1	4,07	54	2177887	2178006	0
0	contig1	4,21	54	2232061	2232168	8
1	contig2	1,48	58	5879	6025	0

RkpS, polysaccharide export protein [Sinorhizobium fredii NGR234]

Hit	Description	E-value	Score	Hit start	Hit end	%Gaps
0	contig1	8,23E-20	224	1673429	1674073	1
0	contig1	4,27E-11	149	2663665	2664273	9
0	contig1	5,62E-10	139	633228	633686	9
0	contig1	1,07E-09	137	1345060	1345629	9
0	contig1	3,62E-08	124	2870144	2870728	13
0	contig1	2,46E-07	117	2651649	2652107	6
0	contig1	6,02E-07	113	2171234	2171875	8
0	contig1	1,27E-05	102	1859546	1859728	3
0	contig1	1,53E-05	101	1440362	1440727	12
0	contig1	2,16E-05	100	1264982	1265491	15
0	contig1	5,23E-05	97	1933205	1933777	9
0	contig1	5,63E-05	96	1238901	1239026	0

0	contig1	6,12E-05	96	3023691	3024290	10
0	contig1	1,17E-04	93	2848359	2848985	13
0	contig1	1,25E-04	93	1706057	1706518	16
0	contig1	1,28E-04	93	1705027	1705743	13
0	contig1	3,71E-04	89	1919031	1919702	14
0	contig1	4,24E-04	89	2000464	2000928	10
0	contig1	4,93E-04	88	570210	570386	0
0	contig1	6,88E-04	87	2508599	2508721	7
0	contig1	8,48E-04	86	2331573	2332211	11
0	contig1	9,37E-04	86	2402851	2403459	12
0	contig1	1,13E-03	85	1030666	1030797	0
0	contig1	2,53E-03	82	571402	572130	14
0	contig1	2,75E-03	82	2006616	2007251	11
0	contig1	3,65E-03	81	2847497	2848135	6
0	contig1	0,01	76	761773	762246	8
0	contig1	0,04	72	1031659	1032030	1
0	contig1	0,04	71	3218638	3219129	13
0	contig1	0,05	71	1818871	1819359	15
0	contig1	0,05	71	2405105	2405230	0
0	contig1	0,05	71	1147051	1147179	0
0	contig1	0,07	70	1932902	1933030	0
0	contig1	0,07	70	700415	701086	11
0	contig1	0,08	69	2869290	2869712	8
0	contig1	0,09	69	2742317	2742430	0
0	contig1	0,13	67	2330917	2331183	2
0	contig1	0,19	66	2008916	2009011	0
0	contig1	0,22	65	227461	227646	0
0	contig1	0,37	63	2176090	2176191	0
0	contig1	0,4	63	3498056	3498115	0

0	contig1	0,81	60	1920061	1920198	4
0	contig1	1,27	59	652158	652256	0
0	contig1	1,41	58	761315	761482	0
0	contig1	1,77	57	3331010	3331135	0
0	contig1	1,86	57	2649777	2649971	23
0	contig1	2,29	56	3220557	3220670	0
0	contig1	2,62	56	2640276	2640362	0
0	contig1	3,17	55	403025	403225	0
0	contig1	4,07	54	2177887	2178006	0
0	contig1	4,21	54	2232061	2232168	8
1	contig2	1,48	58	5879	6025	0

CpxA [Actinobacillus pleuropneumoniae]

Hit	Description	E-value	Score	Hit start	Hit end	%Gaps
0	contig1	2,65E-30	315	1673474	1674028	2
0	contig1	1,03E-18	215	1345003	1345626	8
0	contig1	1,46E-15	187	2663668	2664243	9
0	contig1	3,17E-13	167	2402824	2403441	11
0	contig1	9,54E-13	163	2847479	2848153	7
0	contig1	1,66E-12	161	2000464	2001072	7
0	contig1	8,86E-12	155	3330560	3331135	8
0	contig1	1,11E-11	154	2008406	2009017	12
0	contig1	2,18E-11	151	633270	633845	9
0	contig1	3,10E-11	150	2870150	2870719	7
0	contig1	4,08E-11	149	1859129	1859728	11
0	contig1	3,31E-10	141	571438	572118	11
0	contig1	6,62E-10	139	3218686	3219273	11

0	contig1	1,97E-09	135	1264973	1265704	13
0	contig1	7,02E-09	130	2404640	2405230	14
0	contig1	1,67E-08	127	2171234	2171410	0
0	contig1	1,80E-08	126	1933208	1933825	8
0	contig1	2,69E-08	125	1919022	1919699	13
0	contig1	3,20E-08	124	1238853	1239506	7
0	contig1	3,85E-08	123	2006619	2007230	10
0	contig1	6,57E-08	121	1030663	1031190	24
0	contig1	9,72E-08	120	1705027	1705707	14
0	contig1	1,38E-07	119	1146592	1147227	11
0	contig1	2,83E-07	116	1932455	1933075	9
0	contig1	3,05E-07	116	700466	701089	13
0	contig1	4,19E-07	114	3023625	3024308	9
0	contig1	4,26E-07	114	1686749	1687375	8
0	contig1	5,80E-07	113	2651649	2652287	6
0	contig1	1,07E-06	111	2331558	2332211	11
0	contig1	1,94E-06	109	2848848	2848997	0
0	contig1	3,37E-06	107	2508515	2508727	4
0	contig1	1,51E-05	101	1440602	1440763	0
0	contig1	3,26E-05	98	1818727	1819371	13
0	contig1	1,09E-04	94	1920019	1920681	13
0	contig1	1,57E-04	92	3220575	3221162	11
0	contig1	7,33E-04	87	761611	762207	9
0	contig1	1,63E-03	84	569751	570359	11
0	contig1	2,24E-03	82	2742323	2742457	0
0	contig1	0,01	75	3498056	3498586	8
0	contig1	0,04	72	761306	761404	0
0	contig1	0,05	71	1031659	1031739	0
0	contig1	0,38	63	319865	320239	7

0	contig1	0,38	63	2773944	2774147	6
0	contig1	0,79	60	147217	147441	2
0	contig1	0,84	60	652143	652241	0
0	contig1	1,15	59	1770500	1770628	0
0	contig1	1,85	57	1010730	1010804	0
0	contig1	1,91	57	276388	276522	0
0	contig1	2,03	57	2921797	2921949	2
0	contig1	4,52	54	227440	227505	0
0	contig1	5,66	53	2368013	2368093	0
0	contig1	5,95	53	2919703	2919801	12
0	contig1	6,64	52	2134981	2135040	0
0	contig1	6,98	52	3471180	3471275	0
0	contig1	8,6	51	782656	782751	0

rfbI [Vibrio cholerae]							
Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	9,18E-43	423	1673471	1674073	38	0
0	contig1	2,74E-17	203	1345048	1345689	27	5
0	contig1	2,74E-17	203	2651694	2652281	27	4
0	contig1	4,41E-14	175	2870060	2870740	26	8
0	contig1	4,16E-13	167	2663536	2664255	28	9
0	contig1	1,90E-11	153	2404619	2405287	30	11
0	contig1	2,52E-11	152	1933187	1933780	26	8
0	contig1	6,98E-11	148	633219	633845	26	6
0	contig1	1,90E-10	144	1859066	1859668	25	8
0	contig1	2,03E-10	144	2402782	2403438	26	11
0	contig1	2,30E-10	143	1238877	1239506	25	3
0	contig1	2,38E-10	143	2006583	2007203	29	9
0	contig1	3,03E-10	142	3023658	3024251	26	9

0	contig1	3,89E-10	141	2171291	2171881	24	8
0	contig1	2,61E-09	134	2331564	2332184	27	10
0	contig1	2,84E-09	134	2008385	2009008	28	12
0	contig1	6,16E-09	131	1686749	1687375	26	4
0	contig1	6,26E-09	131	1919073	1919708	27	17
0	contig1	8,39E-09	130	700439	701155	26	14
0	contig1	9,19E-09	130	1818721	1819413	25	9
0	contig1	1,02E-08	129	2847491	2848114	23	5
0	contig1	1,40E-08	128	3330542	3331132	27	7
0	contig1	5,72E-08	123	1146571	1147176	28	12
0	contig1	6,59E-08	122	2000425	2001084	25	7
0	contig1	1,50E-07	119	1920061	1920690	25	11
0	contig1	4,16E-07	115	1264997	1265485	25	15
0	contig1	6,53E-07	114	2848344	2848973	23	13
0	contig1	1,40E-06	111	3220578	3221183	25	14
0	contig1	1,56E-06	110	1932431	1933036	23	9
0	contig1	2,17E-06	109	1440101	1440805	25	6
0	contig1	2,32E-05	100	571474	572100	26	11
0	contig1	3,11E-05	99	2508569	2509162	25	13
0	contig1	3,30E-05	99	2330917	2331153	32	1
0	contig1	4,53E-05	98	1706048	1706719	23	12
0	contig1	2,89E-04	91	1030684	1030845	39	4
0	contig1	8,12E-04	87	1705075	1705749	22	15
0	contig1	1,78E-03	84	761620	762198	21	5
0	contig1	3,25E-03	82	3218731	3219309	23	12
0	contig1	6,38E-03	79	569715	570335	24	10
0	contig1	0,03	74	2509316	2509942	24	11
0	contig1	0,33	64	3498056	3498109	72	0
0	contig1	0,34	64	1954695	1954772	48	0

0	contig1	0,74	61	2368037	2368126	47	0
0	contig1	0,74	61	169264	169356	48	3
0	contig1	0,81	61	2742275	2742421	35	4
0	contig1	0,81	61	1031665	1032114	25	5
0	contig1	2,89	56	761318	761407	37	0
0	contig1	3,06	56	2869326	2869862	21	5
0	contig1	3,87	55	2757025	2757192	36	0
0	contig1	4,17	55	1016515	1016583	48	0
0	contig1	4,77	54	147367	147423	63	0
0	contig1	6,23	53	2177959	2178153	25	1
0	contig1	6,55	53	2649873	2649926	50	0
0	contig1	7,42	53	3138889	3139020	27	0
0	contig1	7,74	53	652143	652241	33	0

malK [Escherichia coli]

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	4,99E-82	763	2847038	2848144	43	3
0	contig1	7,54E-36	365	1686752	1687429	37	3
0	contig1	4,81E-33	341	2000461	2001102	35	6
0	contig1	5,50E-32	332	633213	633896	39	5
0	contig1	2,73E-30	317	2171237	2171884	34	3
0	contig1	2,76E-27	291	1859075	1859671	36	3
0	contig1	2,13E-26	284	3023646	3024305	33	5
0	contig1	1,54E-25	276	1264976	1265650	33	8
0	contig1	2,22E-23	258	700448	701119	32	7
0	contig1	1,27E-22	251	1238847	1239467	29	2
0	contig1	1,36E-22	251	1345000	1345659	31	0
0	contig1	5,42E-22	246	1818712	1819368	28	5
0	contig1	6,97E-21	236	1706048	1706698	29	7

0	contig1	1,01E-20	235	1920037	1920693	30	8
0	contig1	3,99E-20	230	1919025	1919717	30	8
0	contig1	1,28E-19	225	2508506	2509159	26	3
0	contig1	8,31E-19	218	2331546	2332190	30	6
0	contig1	1,14E-18	217	2008361	2009011	33	8
0	contig1	2,34E-18	214	2651652	2652284	28	1
0	contig1	5,48E-18	211	1932431	1933081	28	2
0	contig1	8,24E-18	210	1146484	1147179	29	6
0	contig1	1,05E-17	209	3220530	3221273	26	4
0	contig1	2,74E-17	205	3498035	3498583	33	4
0	contig1	4,45E-16	195	2404574	2405227	32	4
0	contig1	5,08E-16	194	2663632	2664246	29	0
0	contig1	5,76E-16	194	3330539	3331135	35	6
0	contig1	3,99E-15	186	3218674	3219294	27	4
0	contig1	8,05E-15	184	2402836	2403495	27	8
0	contig1	2,88E-14	179	2330503	2331156	28	8
0	contig1	3,16E-14	179	571477	572076	28	3
0	contig1	4,60E-14	177	761581	762186	28	3
0	contig1	6,06E-14	176	2870156	2870956	23	1
0	contig1	1,84E-13	172	2848341	2848970	26	6
0	contig1	5,62E-13	168	2006601	2007218	25	6
0	contig1	6,37E-13	167	1705069	1705725	30	7
0	contig1	1,06E-12	166	1673477	1674064	26	0
0	contig1	9,60E-12	157	569733	570332	27	7
0	contig1	2,40E-11	154	2869248	2869862	28	7
0	contig1	1,44E-10	147	1933205	1933810	26	3
0	contig1	1,23E-09	139	1440152	1440781	27	0
0	contig1	1,92E-07	120	2509331	2509945	24	10
0	contig1	3,33E-07	118	1030624	1031331	23	13

0	contig1	1,89E-04	94	2741945	2742424	23	8
0	contig1	1,92E-04	94	760934	761416	23	0
0	contig1	2,55E-03	85	1031665	1032120	23	1
0	contig1	0,45	65	227452	227538	48	0
0	contig1	0,62	64	2705682	2705738	53	0
0	contig1	0,96	62	2649876	2650028	38	15
0	contig1	1	62	2176114	2176191	50	0
0	contig1	1,19	62	1781792	1781866	52	12
0	contig1	1,36	61	149627	149686	50	0
0	contig1	3,25	58	2177926	2178015	50	0
0	contig1	3,34	58	676124	676192	48	0
0	contig1	3,39	58	2913347	2913463	36	8
0	contig1	3,98	57	1718819	1718941	32	0
0	contig1	6,08	56	3561401	3561502	47	12

ABC transporter, ATP-binding protein/permease, glycan transport [Campylobacter lari RM2100]

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	3,82E-35	361	2006601	2007422	30	3
0	contig1	5,34E-33	343	2404628	2405536	29	1
0	contig1	1,90E-31	329	569739	570296	37	1
0	contig1	2,10E-31	329	2008400	2008969	36	3
0	contig1	2,23E-31	329	1146574	1147134	37	1
0	contig1	4,34E-31	326	571477	572040	34	1
0	contig1	2,79E-29	310	2402836	2403522	31	0
0	contig1	1,36E-28	305	3220617	3221180	35	1
0	contig1	8,61E-28	298	3218494	3219279	30	5
0	contig1	1,63E-17	209	700487	701164	30	5
0	contig1	2,90E-16	198	1859075	1859626	31	4

0	contig1	1,91E-15	191	3023640	3024200	29	4
0	contig1	2,87E-15	190	2171330	2171878	29	5
0	contig1	9,47E-15	185	2331612	2332157	33	6
0	contig1	1,93E-13	174	1686857	1687270	32	4
0	contig1	4,08E-13	171	1706096	1706536	35	11
0	contig1	4,86E-13	170	1920112	1920645	33	7
0	contig1	2,35E-12	164	1818715	1819284	32	5
0	contig1	4,01E-12	162	633312	633704	38	4
0	contig1	1,03E-11	159	3330596	3331084	29	3
0	contig1	1,11E-11	159	2508599	2509168	27	3
0	contig1	1,35E-11	158	2848344	2848931	24	7
0	contig1	4,86E-11	153	2869326	2869865	28	3
0	contig1	6,62E-11	152	2651736	2652251	27	5
0	contig1	6,74E-11	152	1919121	1919612	30	9
0	contig1	7,96E-11	151	3498056	3498475	30	5
0	contig1	9,48E-11	151	2000569	2001066	28	8
0	contig1	1,12E-10	150	1265060	1265605	31	11
0	contig1	2,03E-10	148	1705126	1705719	30	10
0	contig1	3,60E-10	146	2847569	2848051	26	2
0	contig1	4,44E-10	145	2870192	2870719	26	7
0	contig1	5,33E-10	144	1238940	1239407	30	4
0	contig1	1,69E-09	140	2330557	2331162	29	9
0	contig1	7,09E-09	134	1673516	1674034	25	4
0	contig1	2,48E-08	130	1345090	1345632	28	5
0	contig1	4,71E-08	127	1932446	1932988	24	2
0	contig1	6,97E-08	126	761614	762144	25	4
0	contig1	8,03E-08	125	1933208	1933738	26	8
0	contig1	4,05E-06	111	2178520	2178771	30	5
0	contig1	1,72E-05	105	2509361	2509804	24	11

0	contig1	1,22E-03	89	1440158	1440682	24	8
0	contig1	1,41E-03	89	761093	761404	24	11
0	contig1	1,57E-03	88	2663716	2664204	28	6
0	contig1	0,03	77	1031086	1031208	37	0
0	contig1	0,34	68	2741945	2742037	45	0
0	contig1	1,05	64	2177518	2177748	25	4
0	contig1	4,53	59	1031950	1032042	42	0
0	contig1	4,72	58	3467988	3468218	28	6
0	contig1	5	58	73701	73835	38	0
0	contig1	5,96	58	2649879	2649926	63	0

MsbA [Escherichia coli]

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	2,61E-65	621	3219480	3221240	28	4
0	contig1	4,54E-63	602	2006535	2008256	28	1
0	contig1	6,13E-60	575	1146508	1148301	29	5
0	contig1	1,82E-58	562	2402782	2404395	28	2
0	contig1	1,49E-50	494	2008343	2009878	26	2
0	contig1	4,02E-49	482	2404565	2406013	28	2
0	contig1	4,16E-43	430	3217612	3219357	24	1
0	contig1	2,68E-41	414	571426	572979	24	1
0	contig1	2,02E-31	329	569688	571391	22	2
0	contig1	3,50E-14	180	700514	701173	27	8
0	contig1	2,14E-08	130	1705027	1705668	26	8
0	contig1	2,84E-08	129	2869278	2869814	25	0
0	contig1	5,78E-08	127	1686752	1687330	28	7
0	contig1	1,41E-07	123	2171114	2171812	27	3
0	contig1	2,80E-07	121	1238892	1239407	25	4
0	contig1	2,94E-07	121	1859141	1859689	27	5

0	contig1	4,31E-07	119	1919025	1919660	25	13
0	contig1	8,70E-07	117	1818766	1819332	26	7
0	contig1	1,81E-06	114	3023721	3024260	24	3
0	contig1	2,07E-06	113	1264976	1265167	36	0
0	contig1	7,30E-06	109	2870153	2870686	24	7
0	contig1	8,07E-06	108	1673453	1673983	26	2
0	contig1	1,46E-05	106	2848455	2849018	22	7
0	contig1	1,50E-05	106	2000464	2001027	24	2
0	contig1	3,48E-05	103	2742269	2742433	35	4
0	contig1	5,14E-05	101	2508566	2509114	23	3
0	contig1	1,10E-04	98	1440503	1440730	30	0
0	contig1	1,18E-04	98	1706036	1706191	42	2
0	contig1	2,57E-04	95	2331564	2332136	23	9
0	contig1	7,37E-04	91	1933631	1933780	40	0
0	contig1	1,55E-03	89	1920079	1920639	25	10
0	contig1	1,67E-03	88	3330593	3331132	24	3
0	contig1	7,19E-03	83	633261	633368	53	0
0	contig1	8,35E-03	82	2177923	2178021	55	0
0	contig1	0,02	80	2847848	2848099	26	1
0	contig1	0,02	79	2330389	2330745	27	10
0	contig1	0,07	75	2651694	2652188	22	1
0	contig1	0,09	73	2176111	2176194	54	0
0	contig1	0,1	73	1031623	1031748	36	0
0	contig1	0,2	70	1345048	1345224	29	0
0	contig1	0,33	68	706489	706680	33	6
0	contig1	0,38	68	762103	762249	33	0
0	contig1	0,43	68	2663677	2663904	19	0
0	contig1	0,51	67	1030684	1030797	37	0
0	contig1	1,13	64	2368025	2368339	27	4

0	contig1	2,29	61	1063435	1063719	23	2
0	contig1	3,45	60	3403003	3403248	26	0
0	contig1	4,55	59	2711764	2712036	25	9
0	contig1	7,31	57	2796963	2797040	38	0
0	contig1	8,36	56	543747	543989	27	4
0	contig1	9,55	56	1014555	1014851	25	0

TagH [Pseudomonas fluorescens F113]							
Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	4,67E-61	587	1673345	1674049	48	2
0	contig1	3,41E-18	217	1345024	1345716	26	8
0	contig1	5,14E-17	207	2651670	2652290	27	5
0	contig1	2,39E-16	201	1933169	1933822	28	6
0	contig1	1,12E-14	187	1919070	1919789	27	12
0	contig1	8,41E-14	179	2331549	2332208	24	16
0	contig1	5,78E-13	172	633201	633956	26	9
0	contig1	5,93E-13	172	2847488	2848114	25	5
0	contig1	1,75E-12	168	2663677	2664252	30	6
0	contig1	3,54E-12	165	2171258	2171881	26	7
0	contig1	4,58E-12	164	2402821	2403438	30	12
0	contig1	8,85E-12	162	2000485	2001144	29	8
0	contig1	1,39E-11	160	1238868	1239494	24	6
0	contig1	2,18E-11	158	1706054	1706710	24	12
0	contig1	1,22E-10	152	1686791	1687405	27	8
0	contig1	2,82E-10	149	2870138	2870758	26	5
0	contig1	3,25E-09	139	3023610	3024275	25	8
0	contig1	4,51E-09	138	2848329	2848973	26	11
0	contig1	7,19E-09	136	1264997	1265683	25	13
0	contig1	1,03E-08	135	2008376	2009008	25	11

0	contig1	1,09E-08	135	2508512	2509162	26	8
0	contig1	2,04E-08	133	1859072	1859677	24	8
0	contig1	2,13E-08	132	2330515	2331177	23	14
0	contig1	6,19E-08	128	571450	572094	25	9
0	contig1	8,94E-08	127	1920073	1920714	21	10
0	contig1	1,25E-07	126	1818721	1819362	23	10
0	contig1	8,94E-07	118	761620	762198	24	5
0	contig1	3,57E-06	113	1705033	1705740	24	14
0	contig1	5,70E-06	111	2404595	2405284	23	9
0	contig1	9,72E-06	109	700433	701095	24	11
0	contig1	1,74E-05	107	1030684	1030836	43	4
0	contig1	2,20E-05	106	2006586	2007203	24	10
0	contig1	2,50E-05	106	2509235	2510002	23	11
0	contig1	4,82E-05	103	3220578	3221195	23	14
0	contig1	7,20E-05	102	3330521	3331132	21	8
0	contig1	1,11E-04	100	1440602	1440844	32	0
0	contig1	4,26E-04	95	760871	761404	24	1
0	contig1	4,99E-04	95	569718	570335	26	9
0	contig1	8,16E-04	93	2869272	2869775	26	5
0	contig1	5,90E-03	85	3498056	3498610	22	8
0	contig1	9,10E-03	84	1932404	1933045	21	8
0	contig1	9,18E-03	84	1031665	1032129	19	7
0	contig1	0,01	82	1146559	1147176	23	13
0	contig1	0,05	78	2741762	2742421	24	11
0	contig1	0,06	77	1144660	1144986	24	4
0	contig1	0,47	69	2008100	2008378	27	9
0	contig1	0,69	68	3218677	3218826	34	0
0	contig1	1,04	66	2649819	2649926	33	0
0	contig1	1,04	66	598449	598637	38	24

0	contig1	2,5	63	712060	712272	30	11
0	contig1	3,7	61	227452	227514	62	0
0	contig1	4,37	61	1766967	1767056	47	0

KpsT [Escherichia coli]

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	1,38E-32	334	1673468	1674025	37	2
0	contig1	5,15E-12	157	1345033	1345686	25	12
0	contig1	1,19E-11	154	1859075	1859728	26	9
0	contig1	2,13E-11	152	2663674	2664267	26	7
0	contig1	1,73E-10	144	2651649	2652287	24	8
0	contig1	1,12E-09	137	2000464	2001114	24	7
0	contig1	1,58E-08	127	1238880	1239506	23	10
0	contig1	2,11E-08	126	700460	701092	24	14
0	contig1	3,79E-08	123	1440113	1440748	25	6
0	contig1	4,05E-08	123	1919031	1919720	24	14
0	contig1	5,20E-08	122	2847488	2848135	24	7
0	contig1	6,57E-08	121	3330560	3331135	27	7
0	contig1	1,07E-07	120	2171234	2171878	26	10
0	contig1	2,18E-07	117	2008412	2009011	25	10
0	contig1	3,66E-07	115	3023658	3024308	24	9
0	contig1	7,70E-07	112	1705982	1706635	21	18
0	contig1	8,02E-07	112	1264982	1265686	22	13
0	contig1	1,40E-06	110	761617	762246	24	7
0	contig1	1,71E-06	109	633210	633839	26	8
0	contig1	2,37E-06	108	2006613	2007251	23	9
0	contig1	6,09E-06	104	1146586	1147260	22	12
0	contig1	9,25E-06	103	1933196	1933825	21	6
0	contig1	1,16E-05	102	2848326	2849021	22	12

0	contig1	2,22E-05	100	3218638	3219279	24	12
0	contig1	3,23E-05	98	1705027	1705713	21	13
0	contig1	4,19E-05	97	1818703	1819359	22	10
0	contig1	4,79E-05	97	2870144	2870749	23	12
0	contig1	1,06E-04	94	571525	572052	24	11
0	contig1	2,24E-04	91	2330917	2331150	27	3
0	contig1	1,05E-03	85	2404634	2405230	24	12
0	contig1	1,53E-03	84	2176096	2176191	41	0
0	contig1	3,86E-03	80	2508521	2509159	21	11
0	contig1	7,15E-03	78	1932869	1933030	31	4
0	contig1	0,03	73	3498056	3498535	21	11
0	contig1	0,04	72	1030663	1030797	31	0
0	contig1	0,06	70	2402887	2403480	25	11
0	contig1	0,07	70	1920082	1920687	21	14
0	contig1	0,14	67	2869323	2869871	21	8
0	contig1	0,19	66	1156609	1156764	26	0
0	contig1	0,21	65	761315	761422	42	0
0	contig1	0,25	65	2177908	2178006	33	0
0	contig1	0,5	62	1178062	1178127	55	0
0	contig1	0,54	62	618114	618302	34	3
0	contig1	1,02	59	570210	570338	28	0
0	contig1	1,2	59	1997197	1997451	22	3
0	contig1	1,23	59	569787	569969	25	0
0	contig1	1,75	57	2368025	2368093	52	0
0	contig1	2,06	57	312757	312831	46	0
0	contig1	2,21	57	3220536	3220670	31	2
0	contig1	2,5	56	1031665	1031739	36	0
0	contig1	2,7	56	2796969	2797040	50	0
0	contig1	3,18	55	1051559	1051693	33	2

0	contig1	4,12	54	2049818	2049922	34	0
0	contig1	6,31	53	2640294	2640356	52	0
1	contig2	0,25	65	63551	63670	40	0
1	contig2	7,21	52	144513	144578	55	9

Wzt [Escherichia coli]

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
0	contig1	1,83E-51	501	1673468	1674145	46	3
0	contig1	5,76E-12	160	1345057	1345683	26	10
0	contig1	1,41E-09	139	2663656	2664243	26	7
0	contig1	2,67E-09	137	633270	633839	25	6
0	contig1	3,61E-09	136	2651640	2652296	22	7
0	contig1	1,48E-08	131	2870144	2870761	22	4
0	contig1	1,50E-07	122	3330485	3331135	22	7
0	contig1	2,17E-07	120	2331582	2332247	24	12
0	contig1	8,11E-07	116	1265021	1265689	23	13
0	contig1	2,08E-06	112	2509331	2509942	23	14
0	contig1	3,21E-06	110	1686800	1687405	24	6
0	contig1	1,23E-05	105	700433	701161	22	12
0	contig1	1,34E-05	105	2508566	2509159	23	9
0	contig1	3,01E-05	102	1238901	1239515	20	6
0	contig1	3,80E-05	101	1919367	1919705	29	10
0	contig1	3,86E-05	101	2330917	2331150	27	1
0	contig1	3,90E-05	101	1933187	1933777	23	7
0	contig1	3,90E-05	101	2171291	2171887	24	8
0	contig1	4,06E-05	101	3220575	3221204	24	8
0	contig1	5,96E-05	99	1818691	1819377	23	11
0	contig1	7,53E-05	99	2847440	2847892	18	3
0	contig1	1,35E-04	96	2008412	2008636	30	0

0	contig1	1,69E-04	96	2402833	2403441	27	11
0	contig1	4,92E-04	92	3023649	3024248	21	8
0	contig1	6,22E-04	91	1859054	1859671	22	9
0	contig1	6,32E-04	91	2006577	2007206	23	13
0	contig1	8,61E-04	89	571450	572079	23	10
0	contig1	9,52E-04	89	2404634	2405248	25	10
0	contig1	5,18E-03	83	2000854	2001072	29	1
0	contig1	8,54E-03	81	1146559	1147191	22	11
0	contig1	0,01	80	761566	761847	25	0
0	contig1	0,01	80	3498245	3498475	31	12
0	contig1	0,02	78	1440113	1440730	24	6
0	contig1	0,05	74	2848296	2848976	21	11
0	contig1	0,14	70	2869494	2869883	22	2
0	contig1	0,24	68	1705477	1705740	22	1
0	contig1	0,27	68	1932422	1933030	20	9
0	contig1	0,28	68	569709	570338	22	10
0	contig1	0,63	65	1030675	1031331	22	18
0	contig1	2,38	60	2344537	2344719	31	2
0	contig1	8,06	55	3450379	3450477	32	7

10.7. BLAST of selected sequences for RT-PCR against *S. thermosulfidooxidans* genome.

Sulth_1631							
Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
2506213081	Contig 1	0	2.174,00	1673340	1674545	100	0
2506213081	Contig 1	0	2.127,00	1673340	1674545	100	0
2506213081	Contig 1	0	2.107,00	1673339	1674547	99	0

2506213081	Contig 1	0	2.056,00	1673339	1674547	100	0
2506213081	Contig 1	0	2.034,00	1673341	1674546	95	0
2506213081	Contig 1	0	1.140,00	1673890	1674546	100	0
2506213081	Contig 1	0	826	1673341	1673835	100	0
2506213081	Contig 1	4,83E-18	85	2663950	2664093	35	0
2506213081	Contig 1	4,83E-18	84	2663677	2663772	47	0
2506213081	Contig 1	4,83E-18	59	2664154	2664243	33	0
2506213081	Contig 1	4,83E-18	51	2663881	2663940	45	0
2506213081	Contig 1	8,80E-14	96	633438	633704	30	0
2506213081	Contig 1	8,80E-14	91	633273	633413	43	0
2506213081	Contig 1	1,41E-12	92	1345048	1345185	37	0
2506213081	Contig 1	1,41E-12	86	1345261	1345476	29	0
2506213081	Contig 1	7,39E-11	117	2651958	2652284	24	0
2506213081	Contig 1	7,39E-11	102	1265018	1265161	38	0
2506213081	Contig 1	7,39E-11	57	2917047	2917097	47	0
2506213081	Contig 1	7,39E-11	52	1700009	1700077	48	0
2506213081	Contig 1	7,39E-11	50	692203	692247	67	0
2506213081	Contig 1	7,39E-11	48	3411750	3411809	40	0
2506213081	Contig 1	2,65E-09	99	1859531	1859668	48	0
2506213081	Contig 1	2,65E-09	88	1818688	1818951	30	0
2506213081	Contig 1	4,49E-09	119	1440590	1440736	49	0
2506213081	Contig 1	1,60E-08	115	2848812	2848973	41	0
2506213081	Contig 1	2,03E-07	107	1819183	1819377	37	0
2506213081	Contig 1	2,79E-07	106	1933640	1933786	45	0
2506213081	Contig 1	9,93E-07	102	2007066	2007203	46	0
2506213081	Contig 1	1,36E-06	101	2405099	2405233	47	0
2506213081	Contig 1	1,37E-06	101	2000485	2000646	43	0
2506213081	Contig 1	1,82E-06	100	2403301	2403438	43	0
2506213081	Contig 1	1,82E-06	81	700526	700771	27	0

2506213081	Contig 1	1,82E-06	59	321784	321867	50	0
2506213081	Contig 1	1,82E-06	50	576186	576308	29	0
2506213081	Contig 1	1,82E-06	50	285925	285969	53	0
2506213081	Contig 1	1,82E-06	48	2155385	2155423	62	0
2506213081	Contig 1	5,02E-06	87	1932887	1933027	40	0
2506213081	Contig 1	5,02E-06	75	1859132	1859302	28	0
2506213081	Contig 1	1,26E-05	94	1238898	1239032	42	0
2506213081	Contig 1	3,27E-05	91	2008910	2009008	55	0
2506213081	Contig 1	6,18E-05	89	2508569	2508700	39	0
2506213081	Contig 1	8,47E-05	88	3331013	3331132	48	0
2506213081	Contig 1	8,47E-05	88	3024108	3024251	46	0
2506213081	Contig 1	8,47E-05	88	1147051	1147188	39	0
2506213081	Contig 1	8,49E-05	88	3220578	3220715	37	0
2506213081	Contig 1	2,20E-04	85	700976	701077	50	0
2506213081	Contig 1	3,02E-04	84	1859550	1859669	43	0
2506213081	Contig 1	4,15E-04	83	2663682	2663771	53	0
2506213081	Contig 1	4,16E-04	83	3218668	3218826	34	0
2506213081	Contig 1	5,71E-04	82	1705081	1705176	53	0
2506213081	Contig 1	7,85E-04	81	1919079	1919186	47	0
2506213081	Contig 1	5,28E-03	75	2869302	2869424	44	0
2506213081	Contig 1	9,93E-03	73	761674	761979	21	0
2506213081	Contig 1	0,03	70	571948	572082	36	0
2506213081	Contig 1	0,04	66	1031644	1031790	24	0
2506213081	Contig 1	0,04	66	1687034	1687231	32	0
2506213081	Contig 1	0,04	57	2120754	2120834	44	0
2506213081	Contig 1	0,04	53	2331925	2331972	63	0
2506213081	Contig 1	0,05	68	3331008	3331088	52	0
2506213081	Contig 1	0,05	68	2177923	2178021	42	0
2506213081	Contig 1	0,07	67	761321	761458	33	0

2506213081	Contig 1	0,09	66	2008929	2009030	35	0
2506213081	Contig 1	0,13	65	1440591	1440731	40	0
2506213081	Contig 1	0,17	64	2331602	2331715	45	0
2506213081	Contig 1	0,24	63	570198	570335	30	0
2506213081	Contig 1	0,33	62	2848837	2848968	36	0
2506213081	Contig 1	0,45	61	2403308	2403418	30	0
2506213081	Contig 1	0,45	61	173621	173680	45	0
2506213081	Contig 1	0,62	60	2405154	2405237	39	0
2506213081	Contig 1	0,85	59	929522	929617	38	0
2506213081	Contig 1	0,85	59	2403302	2403433	36	0
2506213081	Contig 1	0,85	59	761051	761191	28	0
2506213081	Contig 1	0,85	59	3269528	3269584	53	0
2506213081	Contig 1	1,16	58	3330542	3330670	23	0
2506213081	Contig 1	1,6	57	2649873	2649923	65	0
2506213081	Contig 1	2,2	56	2796957	2797046	33	0
2506213081	Contig 1	2,2	56	415846	415935	47	0
2506213081	Contig 1	3,03	55	1931090	1931173	39	0
2506213081	Contig 1	3,03	55	3498059	3498106	63	0
2506213081	Contig 1	4,15	54	570259	570336	50	0
2506213081	Contig 1	4,15	54	1538475	1538624	26	0
2506213081	Contig 1	4,16	54	2921578	2921739	31	0
2506213081	Contig 1	5,7	53	3462292	3462384	32	0
2506213081	Contig 1	5,7	53	3220622	3220771	28	0
2506213081	Contig 1	5,7	53	2953028	2953069	64	0
2506213081	Contig 1	5,7	53	1147100	1147189	43	0
2506213081	Contig 1	5,7	53	86474	86521	44	0
2506213081	Contig 1	5,71	53	2592570	2592626	42	0
2506213081	Contig 1	7,83	52	370738	370791	50	0
2506213081	Contig 1	7,83	52	3114223	3114306	43	0

2506213081	Contig 1	7,83	52	2566339	2566407	39	0
2506213081	Contig 1	7,83	52	2304152	2304220	48	0
2506213081	Contig 1	7,83	52	1459246	1459392	24	0
2506213081	Contig 1	7,83	52	1797423	1797554	27	0
2506213081	Contig 1	7,83	52	1091383	1091454	46	0
2506213081	Contig 1	7,83	52	2627041	2627133	35	0
2506213081	Contig 1	7,83	52	3246377	3246442	41	0
2506213081	Contig 1	7,85	52	1067348	1067392	53	0

Sulth_1632

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
2506213081	Contig 1	0	2.174,00	1673340	1674545	100	0
2506213081	Contig 1	0	2.127,00	1673340	1674545	100	0
2506213081	Contig 1	0	2.107,00	1673339	1674547	99	0
2506213081	Contig 1	0	2.056,00	1673339	1674547	100	0
2506213081	Contig 1	0	2.034,00	1673341	1674546	95	0
2506213081	Contig 1	0	1.140,00	1673890	1674546	100	0
2506213081	Contig 1	0	826	1673341	1673835	100	0
2506213081	Contig 1	4,83E-18	85	2663950	2664093	35	0
2506213081	Contig 1	4,83E-18	84	2663677	2663772	47	0
2506213081	Contig 1	4,83E-18	59	2664154	2664243	33	0
2506213081	Contig 1	4,83E-18	51	2663881	2663940	45	0
2506213081	Contig 1	8,80E-14	96	633438	633704	30	0
2506213081	Contig 1	8,80E-14	91	633273	633413	43	0
2506213081	Contig 1	1,41E-12	92	1345048	1345185	37	0
2506213081	Contig 1	1,41E-12	86	1345261	1345476	29	0
2506213081	Contig 1	7,39E-11	117	2651958	2652284	24	0
2506213081	Contig 1	7,39E-11	102	1265018	1265161	38	0
2506213081	Contig 1	7,39E-11	57	2917047	2917097	47	0

2506213081	Contig 1	7,39E-11	52	1700009	1700077	48	0
2506213081	Contig 1	7,39E-11	50	692203	692247	67	0
2506213081	Contig 1	7,39E-11	48	3411750	3411809	40	0
2506213081	Contig 1	2,65E-09	99	1859531	1859668	48	0
2506213081	Contig 1	2,65E-09	88	1818688	1818951	30	0
2506213081	Contig 1	4,49E-09	119	1440590	1440736	49	0
2506213081	Contig 1	1,60E-08	115	2848812	2848973	41	0
2506213081	Contig 1	2,03E-07	107	1819183	1819377	37	0
2506213081	Contig 1	2,79E-07	106	1933640	1933786	45	0
2506213081	Contig 1	9,93E-07	102	2007066	2007203	46	0
2506213081	Contig 1	1,36E-06	101	2405099	2405233	47	0
2506213081	Contig 1	1,37E-06	101	2000485	2000646	43	0
2506213081	Contig 1	1,82E-06	100	2403301	2403438	43	0
2506213081	Contig 1	1,82E-06	81	700526	700771	27	0
2506213081	Contig 1	1,82E-06	59	321784	321867	50	0
2506213081	Contig 1	1,82E-06	50	576186	576308	29	0
2506213081	Contig 1	1,82E-06	50	285925	285969	53	0
2506213081	Contig 1	1,82E-06	48	2155385	2155423	62	0
2506213081	Contig 1	5,02E-06	87	1932887	1933027	40	0
2506213081	Contig 1	5,02E-06	75	1859132	1859302	28	0
2506213081	Contig 1	1,26E-05	94	1238898	1239032	42	0
2506213081	Contig 1	3,27E-05	91	2008910	2009008	55	0
2506213081	Contig 1	6,18E-05	89	2508569	2508700	39	0
2506213081	Contig 1	8,47E-05	88	3331013	3331132	48	0
2506213081	Contig 1	8,47E-05	88	3024108	3024251	46	0
2506213081	Contig 1	8,47E-05	88	1147051	1147188	39	0
2506213081	Contig 1	8,49E-05	88	3220578	3220715	37	0
2506213081	Contig 1	2,20E-04	85	700976	701077	50	0
2506213081	Contig 1	3,02E-04	84	1859550	1859669	43	0

2506213081	Contig 1	4,15E-04	83	2663682	2663771	53	0
2506213081	Contig 1	4,16E-04	83	3218668	3218826	34	0
2506213081	Contig 1	5,71E-04	82	1705081	1705176	53	0
2506213081	Contig 1	7,85E-04	81	1919079	1919186	47	0
2506213081	Contig 1	5,28E-03	75	2869302	2869424	44	0
2506213081	Contig 1	9,93E-03	73	761674	761979	21	0
2506213081	Contig 1	0,03	70	571948	572082	36	0
2506213081	Contig 1	0,04	66	1031644	1031790	24	0
2506213081	Contig 1	0,04	66	1687034	1687231	32	0
2506213081	Contig 1	0,04	57	2120754	2120834	44	0
2506213081	Contig 1	0,04	53	2331925	2331972	63	0
2506213081	Contig 1	0,05	68	3331008	3331088	52	0
2506213081	Contig 1	0,05	68	2177923	2178021	42	0
2506213081	Contig 1	0,07	67	761321	761458	33	0
2506213081	Contig 1	0,09	66	2008929	2009030	35	0
2506213081	Contig 1	0,13	65	1440591	1440731	40	0
2506213081	Contig 1	0,17	64	2331602	2331715	45	0
2506213081	Contig 1	0,24	63	570198	570335	30	0
2506213081	Contig 1	0,33	62	2848837	2848968	36	0
2506213081	Contig 1	0,45	61	2403308	2403418	30	0
2506213081	Contig 1	0,45	61	173621	173680	45	0
2506213081	Contig 1	0,62	60	2405154	2405237	39	0
2506213081	Contig 1	0,85	59	929522	929617	38	0
2506213081	Contig 1	0,85	59	2403302	2403433	36	0
2506213081	Contig 1	0,85	59	761051	761191	28	0
2506213081	Contig 1	0,85	59	3269528	3269584	53	0
2506213081	Contig 1	1,16	58	3330542	3330670	23	0
2506213081	Contig 1	1,6	57	2649873	2649923	65	0
2506213081	Contig 1	2,2	56	2796957	2797046	33	0

2506213081	Contig 1	2,2	56	415846	415935	47	0
2506213081	Contig 1	3,03	55	1931090	1931173	39	0
2506213081	Contig 1	3,03	55	3498059	3498106	63	0
2506213081	Contig 1	4,15	54	570259	570336	50	0
2506213081	Contig 1	4,15	54	1538475	1538624	26	0
2506213081	Contig 1	4,16	54	2921578	2921739	31	0
2506213081	Contig 1	5,7	53	3462292	3462384	32	0
2506213081	Contig 1	5,7	53	3220622	3220771	28	0
2506213081	Contig 1	5,7	53	2953028	2953069	64	0
2506213081	Contig 1	5,7	53	1147100	1147189	43	0
2506213081	Contig 1	5,7	53	86474	86521	44	0
2506213081	Contig 1	5,71	53	2592570	2592626	42	0
2506213081	Contig 1	7,83	52	370738	370791	50	0
2506213081	Contig 1	7,83	52	3114223	3114306	43	0
2506213081	Contig 1	7,83	52	2566339	2566407	39	0
2506213081	Contig 1	7,83	52	2304152	2304220	48	0
2506213081	Contig 1	7,83	52	1459246	1459392	24	0
2506213081	Contig 1	7,83	52	1797423	1797554	27	0
2506213081	Contig 1	7,83	52	1091383	1091454	46	0
2506213081	Contig 1	7,83	52	2627041	2627133	35	0
2506213081	Contig 1	7,83	52	3246377	3246442	41	0
2506213081	Contig 1	7,85	52	1067348	1067392	53	0

Sulth_1635

Hit	Description	E-value	Score	Hit start	Hit end	%Identity	%Gaps
2506213081	Contig 1	0	1.659,00	1672432	1673337	100	0
2506213081	Contig 1	0	1.140,00	1672698	1673336	100	0
2506213081	Contig 1	0	431	1672431	1672664	100	0
2506213081	Contig 1	0	1.525,00	1672432	1673337	100	0

2506213081	Contig 1	0	827	1672430	1672915	100	0
2506213081	Contig 1	0	596	1673000	1673338	100	0
2506213081	Contig 1	0	1.358,00	1672431	1673267	96	0
2506213081	Contig 1	0	51	1673304	1673336	100	0
2506213081	Contig 1	1,21E-163	670	1672952	1673338	100	0
2506213081	Contig 1	1,21E-163	610	1672430	1672900	79	0
2506213081	Contig 1	0,03	69	1240451	1240678	26	0
2506213081	Contig 1	0,45	60	414421	414504	43	0
2506213081	Contig 1	0,85	58	168856	168978	34	0
2506213081	Contig 1	0,85	58	1918837	1918929	35	0
2506213081	Contig 1	1,17	57	2883363	2883455	42	0
2506213081	Contig 1	2,19	55	1085153	1085224	46	0
2506213081	Contig 1	2,19	55	377528	377590	48	0
2506213081	Contig 1	2,2	55	273909	274004	31	0
2506213081	Contig 1	2,2	55	579978	580037	55	0
2506213081	Contig 1	3,02	54	2129498	2129566	43	0
2506213081	Contig 1	4,13	53	3606774	3606818	60	0
2506213081	Contig 1	4,13	53	3424718	3424774	42	0
2506213081	Contig 1	4,13	53	603196	603279	39	0
2506213081	Contig 1	4,15	53	999290	999421	32	0
2506213081	Contig 1	4,15	53	2681985	2682077	39	0
2506213081	Contig 1	4,15	53	3263932	3264039	31	0
2506213081	Contig 1	5,68	52	3479720	3479785	50	0
2506213081	Contig 1	5,68	52	3051339	3051401	33	0
2506213081	Contig 1	5,68	52	2427403	2427483	37	0
2506213081	Contig 1	5,68	52	1087840	1087878	62	0
2506213081	Contig 1	5,7	52	302219	302323	43	0
2506213081	Contig 1	5,7	52	1751502	1751549	63	0
2506213081	Contig 1	5,7	52	1753546	1753620	44	0

2506213081	Contig 1	5,7	52	2976419	2976457	69	0
2506213081	Contig 1	7,8	51	3495916	3495972	47	0
2506213081	Contig 1	7,8	51	3172966	3173046	41	0
2506213081	Contig 1	7,8	51	1562493	1562591	42	0
2506213081	Contig 1	7,83	51	473122	473253	32	0
2506213081	Contig 1	7,83	51	796641	796745	34	0
2506213081	Contig 1	7,83	51	1269043	1269093	47	0
2506213081	Contig 1	7,83	51	1770028	1770132	31	0
2506213081	Contig 1	7,83	51	947376	947468	35	0
2506213081	Contig 1	7,83	51	3005142	3005177	67	0
2506213081	Contig 1	7,83	51	3444277	3444348	42	0
2506213081	Contig 1	7,83	51	3606523	3606648	31	0